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(54) Title: IN VIVO AFFINITY MATURATION SCHEME

(57) Abstract: The present invention relates to the field of evolution of nucleic acids in vivo and provides methods and compositions for introducing diversity into gene products. The present invention allows generation of new sequences that have desirable properties by virtue of high frequency mutation events within a cell. The high frequency mutation of a polynucleotide sequence results in the production of a large population of new sequence variants. Appropriate selection and/or screening permits identification and isolation of mutant forms of the polynucleotide sequence as well as products resulting from expression of the mutant sequences.



**WO 2004/055182 A1**

## In vivo affinity maturation scheme

### Field of the Invention

5 The present invention relates to the field of evolution of nucleic acids *in vivo* and provides methods and compositions for introducing diversity into gene products. The present invention allows generation of new sequences that have desirable properties by virtue of high frequency mutation events within a cell. The high frequency mutation of a polynucleotide sequence results in the production of a large population of new  
10 sequence variants. Appropriate selection and/or screening permits identification and isolation of mutant forms of the polynucleotide sequence as well as products resulting from expression of the mutant sequences.

### Background of the Invention

15 *In vitro* evolution of proteins involves generating diversity by introducing mutations into known gene sequences to produce a library of mutant sequences, translating the sequences to produce a very large number of mutant gene products, which are then selected for the desired properties. Such schemes can be divided into two distinct  
20 groups; i) partial *in vitro* methods in which the mutated target protein is displayed on the surface of the either phage, bacteria or yeast but the mutation step is performed outside the cell and ii) entirely *in vitro* methods in which the target is displayed as part of a ribosome quaternary complex or polysome and all other steps including mutation are performed in a cell free environment. These processes have the potential for  
25 generating proteins with improved diagnostic and therapeutic utilities. Unfortunately, however, the potential of such processes has been limited by deficiencies in methods currently available for mutation, library generation and display of correctly folded proteins.

30 For example, in the case of partial *in vitro* methods, the DNA must be synthesised *in vitro* or extracted from the cells for mutagenesis. Although various mutagenesis approaches (including error prone PCR, DNA shuffling, chain shuffling and site directed mutagenesis) have been successfully used to generate mutant libraries, some of this diversity is lost due to limitations in subsequent transformation efficiency.  
35 Consequently, the generation of large libraries (e.g. beyond a library size of  $10^{10}$ ) of unique individual genes and their encoded proteins has proven difficult particularly

with phage display systems. A further disadvantage is that methods which utilise phage display systems require several sequential steps of mutation, amplification, selection and further mutation. Given that extraction and reintroduction of DNA into the cell is required for these systems, their potential to generate large diversity in the target gene library is further restricted.

To circumvent this problem, entirely *in vitro* methods such as continuous *in vitro* evolution (CIVE) have been developed and are described, for example, in WO 99/58661. In theory any diversity created through mutagenesis in these systems is not lost. In the case of CIVE, a mutating enzyme is used to introduce nucleic acid base changes into the target sequence. The only factor limiting diversity here is the mutation rate of this enzyme. Reports of mutation rates using other *in vitro* mutation methods such as error prone PCR, DNA shuffling (sexual PCR), chain shuffling and site directed mutagenesis over selected CDRs which can be used in this scheme vary significantly. Despite using different mechanisms all these approaches operate in an artificial environment in which only defined components required for these processes are present. It is possible there may be additional unknown factors involved, which are not supplied. Furthermore, this cell-free environment lacks the secretory and post-translational machinery required to produce a correctly folded and processed protein. As a result, this restricts the type of targets which can be "evolved" in these systems and allows incorrectly folded, unmodified mutant proteins which have no functional relevance in a clinical setting to be selected. Bacterial and phage display also have the same associated problems.

*In vivo* evolution of proteins involves the same steps and principles as previously described for *in vitro* evolution (i.e. mutation, display, selection and amplification). However, *in vivo* systems overcome many of the problems associated with the *in vitro* approaches. One *in vivo* cyclical procedure that has been reported involves *Escherichia coli* mutator cells that were used as a vehicle for mutation of recombinant antibody genes. The *E.coli* mutator cells, MUTD5-FIT which carried a mutated DNAQ gene were used as the source of the S-30 extracts and therefore allowed mutations to be introduced into DNA during replication as a result of proofreading errors. However, mutation rates were low compared to the required rate. For example, to mutate 20 residues with the complete permutation of 20 amino acid requires a library size of  $1 \times 10^{26}$ , an extremely difficult task with currently available phage library display



methodology. Obviously, the disadvantages with using bacteria and phage in terms of transformation efficiencies, and protein folding etc. make this a less desirable scheme.

5 In view of the above it is clear that the current affinity maturation schemes are somewhat limited in their ability to generate and select functionally superior binders.

#### Summary of the invention

10 The present inventors have now developed a novel process for the *in vivo* evolution of gene products. This process generates mutants of target nucleic acid sequences by somatic hypermutation, yielding mutant products capable of undergoing any post-translational modifications that may be required for biological activity. A selection system particular to the properties of the target product is then utilized to identify desirable mutants.

15

Accordingly, in a first aspect, the present invention provides a method for producing and selecting a gene product with desired characteristics, the method comprising

20 (i) introducing into a hypermutating cell a target nucleic acid molecule encoding a gene product such that the target nucleic acid molecule is integrated into an immunoglobulin locus of the genome of the hypermutating cell;

25 (ii) culturing the hypermutating cell such that the target nucleic acid molecule undergoes hypermutation during DNA and/or RNA synthesis, giving rise to a population of cells expressing mutant gene products; and

(iii) selecting a mutant gene product with desired characteristics.

30 The term "immunoglobulin locus" refers to a variable region of an antibody molecule or all or a portion of a regulatory nucleotide sequence that controls expression of an antibody molecule. Immunoglobulin loci for heavy chains may include but are not limited to all or a portion of the V, D, J, and switch regions (including intervening sequences called introns) and flanking sequences associated with or adjacent to the particular heavy chain constant region gene expressed by the antibody-producing cell to  
35 be transfected and may include regions located within or downstream of the constant region (including introns). Immunoglobulin loci for light chains may include but are

not limited to the V and J regions, their upstream flanking sequences, and intervening sequences (introns), associated with or adjacent to the light chain constant region gene expressed by the antibody-producing cell to be transfected and may include regions located within or downstream of the constant region (including introns).

5 Immunoglobulin loci for heavy chain variable regions may include but are not limited to all or a portion of the V, D, and J regions (including introns) and flanking sequences associated with or adjacent to the particular variable region gene expressed by the antibody-producing cell to be transfected. Immunoglobulin loci for light chain variable regions may include but are not limited to the V and J region (including introns) and

10 flanking sequences associated with or adjacent to the light chain variable region gene expressed by the antibody-producing cell to be transfected.

In the human, the immunoglobulin heavy chain (IgH) locus is located on chromosome 14. In the 5'-3' direction of transcription, the locus comprises a large cluster of variable

15 region genes ( $V_H$ ), the diversity (D) region genes, followed by the joining ( $J_H$ ) region genes and the constant ( $C_H$ ) gene cluster. The size of the locus is estimated to be about from 1,500 to about 2,500 kilobases (kb). During B-cell development, discontinuous gene segments from the germ line IgH locus are juxtaposed by means of a physical rearrangement of the DNA. In order for a functional heavy chain Ig polypeptide to be

20 produced, three discontinuous DNA segments, from the  $V_H$ , D, and  $J_H$  regions must be joined in a specific sequential fashion; first D to  $J_H$  then  $V_H$  to  $DJ_H$ , generating the functional unit  $V_HDJ_H$ . Once a  $V_HDJ_H$  has been formed, specific heavy chains are produced following transcription of the Ig locus, utilizing as a template the specific  $V_HDJ_HC_H$  unit comprising exons and introns.

25 There are two loci for immunoglobulin light chains (IgL), the kappa locus on human chromosome 2 and the lambda locus on human chromosome 22. The organization of the IgL loci is similar to that of the IgH locus, except that the D region is not present. Following IgH rearrangement, rearrangement of a light chain locus is similarly

30 accomplished by  $V_L$  to  $J_L$  joining of the kappa or lambda chain. The sizes of the lambda and kappa loci are each approximately 1000 kb to 2000 kb. Expression of rearranged IgH and an Ig kappa or Ig lambda light chain in a particular B-cell allows for the generation of antibody molecules.

In a further preferred embodiment of the invention the immunoglobulin locus is a rearranged  $V_H4$  gene. In a further preferred embodiment, the immunoglobulin locus is a rearranged  $VH_{4-34}$  allele.

- 5 By "hypermutation" we mean a mechanism by which mutagenesis occurs at a rate approaching that naturally occurring in the immunoglobulin variable region, which is preferably in the range of  $10^{-4}$  to  $10^{-3}$ /base pair/generation/cell but more preferably in the range of  $5 \times 10^{-5}$  to  $5 \times 10^{-4}$ /base pair/generation/cell.

- 10 A "hypermutating cell" is a cell or cell line containing hypermutation elements.

By "hypermutation elements" we mean an intronic enhancer (Ei), matrix attachment regions (MAR), and a 3' enhancer. The intronic enhancer may be, for example, E mu or E kappa. The 3' enhancer may be, for example, a 3' kappa enhancer or a 3'H  
15 enhancer.

A "matrix attachment region" (MAR) is defined by its ability to bind to the nuclear matrix. Matrix attachment region sequences flank the IgH intronic enhancer.

- 20 In one embodiment the hypermutating cell is an immunoglobulin-expressing cell which is capable of expressing at least one immunoglobulin V gene. A V gene may be a variable light chain ( $V_L$ ) or a variable heavy chain ( $V_H$ ) gene, and may be produced as part of an entire immunoglobulin molecule. Preferred hypermutating cells for use in the present invention are derived from B-cell lines. Lymphoma cells may be used for  
25 the isolation of constitutively hypermutating cell lines for use in the present invention.

In a preferred embodiment of the present invention, following integration into the immunoglobulin locus of the hypermutating cell, the target nucleic acid molecule is located in proximity to one or more endogenous hypermutation elements. Preferably,  
30 the immunoglobulin locus is a  $V_H$  gene and the target nucleic acid molecule is located in proximity to an endogenous intronic enhancer, and endogenous matrix attachment regions.

The phrase "located in proximity to" means that hypermutation elements are located  
35 close enough to the target nucleic acid molecule to effect hypermutation of the target nucleic acid molecule.

In an alternative embodiment of the invention, following integration into the immunoglobulin locus of the hypermutating cell, the target nucleic acid molecule is located in proximity to at least one exogenous hypermutation element. For example, the target nucleic acid molecule may be located in proximity to an exogenous intronic enhancer, an exogenous matrix attachment region and/or an exogenous 3' kappa enhancer. Any one or more of these exogenous elements may be integrated into the immunoglobulin locus simultaneously with the target nucleic acid molecule.

A suitable exogenous "intronic enhancer" may be, for example, the *XbaI-EcoRI* fragment described in Grosschedl *et al* (1985) Cell Vol 41:885-897, the intronic enhancer described in Rabbitts *et al* (1983) Nature 306 (5945):806-809, or the intronic enhancer described in Ravetch *et al* (1981) Cell 27 (3 Pt 2): 583-591; or can be one or more sub-fragments thereof determined to have hypermutation activity.

A suitable exogenous "3' kappa enhancer" is the *ScaI-XbaI* fragment described in Meyer *et al* (1989) EMBO Journal Vol. 8, no. 7 p. 1959-1964 and can be one or more sub-fragments determined to have hypermutation activity.

Hypermutation-competent fragments of exogenous intronic enhancers or the 3' kappa enhancer can be identified in a number of ways. One way is to perform deletional analysis by constructing hypermutation cassettes containing various enhancer deletion mutants and a reporter gene. The hypermutation efficiency of the enhancer deletion mutant can be assessed by determining the rate of mutation of the reporter gene. Deletion mutants can be prepared in a variety of ways. Oligonucleotides can be designed containing fragment sequences to be tested. Alternatively, a more random approach is to linearize the expression vector by restriction digest within an enhancer, followed by subsequent exonuclease treatment and religation. Yet another method is to simply use restriction digests to remove sections of DNA.

It is preferred that following integration, a 3' enhancer and/or an intronic enhancer are positioned at a location 3' of the target nucleic acid sequence. It is further preferred that the intronic enhancer be located in greater proximity to the target gene than the 3' enhancer. The 5' end of the intronic enhancer is preferably positioned up to 3 kb 3' of the 3' end of the target gene, preferably less than 2 kb, more preferably less than 1 kb, and most preferably immediately adjacent to the target nucleic acid sequence. The

intronic enhancer can be positioned greater than 3 kb 3' of the target gene, but this is less preferred. The 3' enhancer is preferably located up to 20 kb and preferably 5-15 kb 3' of the intronic enhancer. The 3' enhancer can be located as close as 1 kb 3' of the intronic enhancer, but this is less preferred. In another embodiment, the 3' enhancer fragment is located 5' relative to the target gene. The intronic enhancer can also be positioned 5' relative to the target gene, although this embodiment is less preferred.

In a further preferred embodiment, the enhancers are present in a genomic orientation. The enhancer sequence present in the genomic immunoglobulin gene is present in a "genomic orientation". If it is flipped in the construct so that it now appears in a 3' to 5' orientation (as opposed to the 5' to 3' orientation in the native genomic configuration), it is present in the "reverse orientation". However, the 3' enhancer can be present in reverse orientation. The enhancer can also be present in reverse orientation, but this is less preferred.

In a further preferred embodiment of the first aspect, following integration of the target nucleic acid molecule into the immunoglobulin locus, the target nucleic acid molecule is operatively linked to a promoter. Preferably, the target nucleic acid molecule is located downstream of the promoter and upstream of an intronic enhancer.

The term "promoter" is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers.

In one preferred embodiment of the invention, the promoter is a naturally occurring promoter that exists within the immunoglobulin locus. In one embodiment, the promoter is an immunoglobulin heavy or light chain promoter. Preferably, the promoter is an immunoglobulin heavy chain promoter of a V<sub>H</sub>4 allele. There is strong conservation between the promoter sequences of the V<sub>H</sub>4 alleles (Figure 1). A wide range of hypermutating cell lines (including RAMOS and BL2 cell lines) carry rearranged V<sub>H</sub>4 alleles.

Alternatively, the promoter may be a heterologous or exogenous promoter selected from those which are functional in mammalian cells, although prokaryotic promoters and promoters functional in other eukaryotic cells may be used. The promoter may be derived from promoter sequences of viral or eukaryotic genes. For example, it may be a

promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of  $\alpha$ -actin,  $\beta$ -actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). They may also  
5 be promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter. In a preferred embodiment, the promoter is an immunoglobulin promoter sequence such as  
10 a murine immunoglobulin promoter sequence or a human immunoglobulin heavy chain promoter.

The promoter region of mammalian/human genes can contain several regulatory elements in the DNA sequences, and span several hundred bases or more, it is generally  
15 observed that one of these elements, designated "TATA box" sequence, in eukaryotic promoter regions is usually found approximately 300 bases or more upstream of the translation initiation site (start) sequence, ATG. Rearrangements which bring the V gene promoter into closer proximity to the ATG translation start signal also brings the promoter closer to the enhancer which is 3' and located in the C region. This activates  
20 the promoter which indicates that the close proximity to the enhancer may affect the rate at which the DNA in the VH locus is mutated.

The present inventors have found that in a RAMOS RA-1 cell line in which the rearrangement generates a functional VH4-34 allele, the promoter is significantly closer  
25 to the translation initiation start site than in other mammalian/human genes (based upon the number of nucleotides between the 3' end of the "TATA box" and the initiation codon of the immunoglobulin leader sequence). This proximity of the promoter to the start codon and the three dimensional structure of the promoter caused significant difficulties in cloning this region from RAMOS RA-1.

30 Accordingly, in a further preferred embodiment of the invention, following integration of the target nucleic acid molecule into the immunoglobulin locus, the target nucleic acid molecule is located within close proximity to the promoter. Preferably, the initiation codon of the target nucleic acid molecule is located within 500 bp of the 3'  
35 end of the promoter, more preferably within 200 bp of the 3' end of the promoter, more

preferably within 100 bp of the 3' end of the promoter and more preferably within 20 bp of the 3' end of the promoter.

5 It may also be advantageous for the promoters to be inducible so that the levels of expression of the heterologous gene can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

10 In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

15 In a further preferred embodiment of the present invention, the target nucleic acid molecule is introduced into the cell by way of an integration vector comprising a sequence homologous to a region of at least 500 bp, more preferably at least 2 kb, more preferably approximately 5 kb upstream of a rearranged V gene and a sequence homologous to a region of at least 500 bp, more preferably at least 2 kb, more preferably approximately 5kb downstream of the same rearranged V gene. Preferably, 20 the sequence homologous to a region downstream of the rearranged V gene comprises an intronic enhancer and matrix attachment regions or portions thereof. It is preferred that the upstream and downstream homologous sequences are at least 500 bp in length, more preferably about 5 kb in length.

25 A "target nucleic acid molecule" can be any nucleic acid molecule of interest (including DNA and RNA molecules) encoding a gene product where diversification of the gene product is desired.

30 The "gene product" may be any biologically active molecule of interest. For example, the gene product may be a catalytic molecule such as a ribozyme, a DNzyme, an LNzyme or an RNAi/siRNA molecule. Alternatively, the gene product may be an antibody or fragment thereof, an enzyme, a hormone, a receptor, a cell surface molecule, a viral protein, transcription factor or any other biologically active polypeptide.

35

The selected mutant target sequence may be recycled through the methods of the present invention in order to introduce further diversification. Accordingly, in a further preferred embodiment of the first and second aspects, at least steps (ii) and (iii) of the method are repeated.

5

The hypermutating cell may be a mammalian, avian, yeast, fungi, insect or bacterial cell. In a preferred embodiment, the hypermutating cell is a mammalian cell. The mammalian hypermutating cell may be selected from the group consisting of RAMOS, BL2, BL41, BL70 and Nalm.

10

The method of the present invention may include further steps to increase the rate of mutation of the target nucleic acid sequence. For example, the hypermutating cell may be cultured in the presence of chemical mutagens. Suitable chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, hydrazine or formic acid. Other agents which are analogues of nucleotide or nucleoside precursors include nitrosoguanidine, ribavirin, 5-bromouracil, 2-aminopurine, 5-formyl uridine, isoguanosine, acridine and of N<sup>4</sup>-aminocytidine, N<sup>1</sup>-methyl-N<sup>4</sup>-aminocytidine, 3,N<sup>4</sup>-ethenocytidine, 3-methylcytidine, 5-hydroxycytidine, N<sup>4</sup>-dimethylcytidine, 5-(2-hydroxyethyl)cytidine, 5-chlorocytidine, 5-bromocytidine, N<sup>4</sup>-methyl-N<sup>sup</sup>.4-aminocytidine, 5-aminocytidine, 5-nitrosocytidine, 5-(hydroxyalkyl)-cytidine, 5-(thioalkyl)-cytidine and cytidine glycol, 5-hydroxyuridine, 3-hydroxyethyluridine, 3-methyluridine, O<sup>2</sup>-methyluridine, O<sup>2</sup>-ethyluridine, 5-aminouridine, O<sup>4</sup>-methyluridine, O<sup>4</sup>-ethyluridine, O<sup>4</sup>-isobutyluridine, O<sup>4</sup>-alkyluridine, 5-nitrosouridine, 5-(hydroxyalkyl)-uridine, and 5-(thioalkyl)-uridine, 1,N<sup>6</sup>-ethenoadenosine, 3-methyladenosine, and N<sup>6</sup>-methyladenosine, 8-hydroxyguanosine, O<sup>6</sup>-methylguanosine, O<sup>6</sup>-ethylguanosine, O<sup>6</sup>-isopropylguanosine, 3,N<sup>2</sup>-ethenoguanosine, O<sup>6</sup>-alkylguanosine, 8-oxo-guanosine, 2,N<sup>3</sup>-ethenoguanosine, and 8-aminoguanosine as well as derivatives/analogues thereof. Examples of suitable nucleoside precursors, and synthesis thereof, are described in further detail in USSN 20030119764. Generally, these agents are added to the replication or transcription reaction thereby mutating the sequence. Intercalating agents such as proflavine, acriflavine, quinacrine and the like can also be used.

Random mutagenesis of the target nucleic acid molecule can also be achieved by irradiation with X-rays or ultraviolet light.

35



Antigen stimulation of the hypermutating cells, or exposure of the cells to interleukins (such as IL-2, IL-4 or IL-10) or CD40 ligand or B cell activating factor (BAFF) may also be used to increase the mutation frequency.

5 In one preferred embodiment, the level or activity of activation-induced cytidine deaminase (AID) within the hypermutating cell is increased. This may be achieved, for example, by increasing expression levels of AID within the hypermutating cells. For example, the hypermutating cells may be transfected with plasmid vectors which encode and express AID.

10

It will be appreciated by those skilled in the art that any process of selecting a mutant product can be used in the methods of the present invention.

15

In one embodiment, selection can be achieved by binding to a target molecule or by measurement of a biological response affected by the mutant product.

20

In another example, if the product of interest is an agent that promotes or reduces cell growth or division, the selection process can involve exposing mutant products to a population of cells and monitoring the biological responses of those cells.

In another example, if the mutant product is a receptor ligand, the process can involve exposing mutant proteins to cells expressing the receptor and monitoring a biological response effected by signalling of the receptor.

25

In one embodiment, the mutant product is selected by way of an assay performed within the hypermutating cell. For example, if the target nucleotide sequence encodes an enzyme, the assay may simply measure enzymatic activity.

30

Alternatively, the assay performed within the hypermutating cell may be a protein-fragment complementation assay (PCA). PCAs rely on the complementation of enzyme fragments fused to interacting proteins that reconstitute enzymatic activity once dimerised. Examples of PCA protocols are described in Michnick (2001) *Current opinion in structural biology* 11:472-477; Wehrman *et al.* (2002). *PNAS* 99(6):3469-3474; and Galarneau *et al.* (2002). *Nature* 20:616-622.

35

Suitable enzyme fragments may be derived, for example, from  $\beta$ -lactamase. Studies using the  $\beta$ -lactamase system in both bacteria and mammalian cells have successfully validated it as a suitable reporter system to detect protein-protein interactions inside the cell.

5

The target nucleic acid molecule may therefore encode a fusion protein comprising a binding partner and a  $\beta$ -lactamase fragment. Its binding partner may be introduced into the cell as a fusion protein comprising a complementary  $\beta$ -lactamase fragment. Selection occurs inside the cell with the binding of the target protein to its cognate partner which is detected by  $\beta$ -lactamase activity on a substrate supplied.

10

If selection assays such as PCAs are used, it is not necessary to display the target polypeptide on the surface of the hypermutating cell. These selection assays are particularly advantageous for targets which are naturally found intracellularly.

15

In an alternative embodiment of the present invention, the target nucleic acid molecule is linked to a sequence encoding an anchor domain such that following expression, the mutant gene product is displayed on the surface of the hypermutating cell. Examples of suitable anchor domains include attachment signals from glycosylphosphatidylinositol (GPI) anchored membrane proteins or transmembrane domains of other cell surface proteins.

20

If the gene product to be displayed is not normally found on the cell surface, it is preferred that the target nucleic acid molecule is also linked to a sequence encoding an N-terminal signal peptide (or a leader peptide). This signal peptide facilitates targeting of the gene product to the plasma membrane.

25

Following display on the surface of the hypermutating cell, the mutant gene product may be selected by detecting binding of a binding partner to the mutant gene product. This may involve, for example, labelling cells with a detectable marker such as a fluorescent dye and allowing binding to occur between the mutant protein on the cell surface and its binding partner. If the binding partner has been immobilized on a plate, a suitable detection system, such as a fluorimeter, can be used to identify wells containing a mutant of interest.

30

35

Alternatively, the binding partner may be labelled with a fluorescent tag, and cells expressing a mutant gene product of interest may be sorted using flow cytometric techniques. The binding partner may be selected from the group consisting of an antibody, receptor, hormone, enzyme, cell surface molecule, transcription factor, DNA or RNA molecule.

In another embodiment of the invention, the target gene product is expressed in soluble form.

To prevent repeated mutation after selection, hypermutation may be arrested prior to culturing the selected cells. This can be accomplished in a number of ways, including fusion to a myeloma or repression of an inducible promoter.

In a further preferred embodiment of the invention, the mutant nucleic acid sequence encoding the selected gene product is recovered from the hypermutating cell. This recovery can be achieved by amplification of the mutant nucleic acid sequence in whole or in part by polymerase chain reaction, using oligonucleotides that will anneal to locations outside the region of hypermutation or within the target sequence itself. Alternatively, the mutant nucleic acid sequence may be amplified using RT-PCR. The mutant nucleic acid sequence can then be subcloned for other purposes, such as expression, purification, or characterization.

The conditioned media from the transfected cells can be concentrated if desired and applied to the selection system. Specific binders can be identified directly or indirectly, for example by antibody recognition of either the target gene product itself or an attached tag sequence. The mutant gene products of interest can then be further characterized by a number of protein chemistry techniques such as micro-sequencing.

In a second aspect the present invention provides a gene product produced by a method of the first aspect.

In a third aspect the present invention provides a vector for targeted integration into an immunoglobulin locus of a hypermutating cell, the vector comprising a sequence homologous to a region upstream of a rearranged V gene of the hypermutating cell, a sequence homologous to a region downstream of a rearranged V gene of the hypermutating cell and a site for insertion of a target nucleic acid molecule.

In a preferred embodiment of the third aspect, the region upstream of the rearranged VH gene of the hypermutating cell is a region within nucleotides 1 to 5190 of SEQ ID NO:1. Preferably, the region is at least 500 bp, more preferably at least 2 kb, more preferably about 5 kb within nucleotides 1 to 5190 of SEQ ID NO:1. In a further preferred embodiment the region upstream of the rearranged V gene of the hypermutating cell comprises nucleotides 191 to 5190 of SEQ ID NO:1.

In a further preferred embodiment of the third aspect, the region downstream of the rearranged VH gene of the hypermutating cell is a region within nucleotides 5709 to 8699 of SEQ ID NO:1. Preferably, the region is at least 500 bp, more preferably at least 3 kb, 5709 to 8699 of SEQ ID NO:1. In a further preferred embodiment the region downstream of the rearranged VH gene of the hypermutating cell comprises nucleotides 5709 to 8634 of SEQ ID NO:1.

In a further preferred embodiment of the third aspect, the vector further comprises a selectable marker. The term "selection marker" or "selectable marker" includes both positive and negative selection markers. A "positive selection marker" is a nucleic acid sequence that allows the survival of cells containing the positive selection marker under growth conditions that kill or prevent growth of cells lacking the marker. An example of a positive selection marker is a nucleic acid sequence which promotes expression of the neomycin resistance gene, or the kanamycin resistance gene. Cells not containing the neomycin resistance gene are selected against by application of G418, whereas cells expressing the neomycin resistance gene are not harmed by G418 (positive selection). A "negative selection marker" is a nucleic acid sequence that kills, prevents growth of or otherwise selects against cells containing the negative selection marker, usually upon application of an appropriate exogenous agent. An example of a negative selection marker is a nucleic acid sequence which promotes expression of the thymidine kinase gene of herpes simplex virus (HSV-TK). Cells expressing HSV-TK are selected against by application of ganciclovir or 1-2'-deoxy-2'-fluoro-b-D-arabinofuranosyl-5-iodouracil (FIAU); negative selection), whereas cells not expressing the gene are relatively unharmed by ganciclovir or FIAU.

In a further preferred embodiment the vector encodes an anchor molecule suitable for display of the protein encoded by the target nucleic acid molecule.

In a further preferred embodiment the vector comprises a target nucleic acid molecule with an epitope tag(s) (for example, two flag tags).

5 In a further preferred embodiment, the vector for targeted integration comprises a sequence as set out in SEQ ID NO:110.

10 The present invention provides a novel approach for generating diversity in a gene product (see Figure 2). An important feature of the present invention is that the target nucleic acid molecule is integrated into the immunoglobulin locus of a host cell genome. This integration is achieved by including sequences homologous to regions upstream and downstream of the rearranged V gene in the integration vector. This directed integration ensures that the target nucleic acid molecule is positioned in proximity to elements that effect hypermutation (eg. in proximity to elements such as an intronic enhancer, matrix attachment regions and/or a 3' enhancer) following  
15 integration.

The integration process also ensures that only one copy of the target nucleic acid molecule is present in each cell. This facilitates the recovery of mutant target nucleic acid sequences of interest following the selection process by, for example, PCR or RT-PCR. In contrast, methods which involve the introduction of a target nucleic acid on a  
20 self replication vector or on a vector which integrates randomly into the host genome are likely to result in multiple mutated copies of target nucleic acid molecule in the host cell. This makes it difficult to determine which sequence encodes the mutant gene product which has been selected for its desired properties.

25 It will be appreciated that the methods of the present invention may be used for a variety of purposes. For example, the methods of the present invention can be used to effect affinity maturation of antibodies. In one aspect, the invention may be applied toward improving the affinity of antibodies from "naive," i.e., non-immune, phage  
30 human antibody libraries. Such libraries already exist and yield antibodies to any antigen. However, since they are made from non-immunized individuals, their affinities are low. In another aspect of the invention, the affinity of antibodies that were generated by conventional hybridoma techniques can be improved by applying a high rate mutagenesis system of the invention to the isolated target nucleic acid  
35 encoding for the initial low-affinity antibody. These enhanced-affinity antibodies can

be utilized as improvements over many antibody-based diagnostics and therapeutics currently available.

5 The methods of the present invention allow a very large library of peptides and single-chain antibodies to be screened and the polynucleotide sequence encoding the desired peptide(s) or single-chain antibodies to be selected. The pool of polynucleotides can then be isolated and shuffled to recombine combinatorially the amino acid sequence of the selected peptide(s) (or predetermined portions thereof) or single-chain antibodies (or just V<sub>H</sub>, V<sub>L</sub>, or CDR portions thereof). Using these methods, one can identify a  
10 peptide or single-chain antibody as having a desired binding affinity for a molecule and can exploit the process of the invention to converge rapidly to a desired high-affinity peptide or scFv. The peptide or antibody can then be synthesized in bulk by conventional means for any suitable use (e.g., as a therapeutic or diagnostic agent).

15 The mutagenesis system can also be used to effect receptor or ligand modification. In one aspect, the invention can generate a ligand or receptor with enhanced binding characteristics for its corresponding receptor or ligand. In another aspect, the mutagenesis system can be used to generate an inhibitor of functional receptor-ligand interaction by creating a ligand or receptor that still binds, but does not elicit a  
20 functional response. In yet another aspect of the invention, multiple biologically active variants of a target protein can be identified and recovered, thereby providing a means to study structure-function relationships of the protein. Additionally, species diversity can be investigated by comparing results obtained by selections utilizing receptors or other molecules from different species.

25 A receptor or ligand can be modified such that it can still bind, but does not signal any more. Alternatively, a better signalling ligand can be selected, which would provide a lower effective dosage of a pharmacologically active therapeutic.

30 The mutagenesis system of the present invention may also be used, for example, on a target such as caspase, an initiation factor target involved in a novel survival mechanism. This involves a cascade of essentially signalling reactions on the route to programmed cell death (apoptosis). Caspase-3 once activated binds to, and cleaves (activates) the 'cell death' proteins (including Id3). *In vivo* mutation and expression of  
35 mutated caspases, especially caspase-3, would have an effect on apoptosis. Therefore caspase 3 would be a preferred target molecule. This could be relevant to diagnostics

in cell signal transduction for monitoring and detection of cancer, and with potential therapeutic outcomes.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

#### A Brief Description of the Figures

10

**Figure 1:** Alignment of human immunoglobulin heavy chain promoters for VH4 alleles. Sequences provided as SEQ ID NO's 2 to 7.

15

**Figure 2:** Schematic representation of a preferred method of the present invention.

**Figure 3:** Schematic representation of the gene targeting region - the preferred site for direct insertion of the target nucleic acid into a rearranged V gene.

20

**Figure 4:** Schematic representation of vector 3kb15a-7-4T.

**Figure 5:** Schematic representation of vector KW2.

**Figure 6:** Schematic representation of vector for targeted integration, KW3.

25

**Figure 7:** RAMOS cells stained with CFSE showing successive divisions on each day.

**Figure 8:** Schematic representation of vector pME18SasFP499.

30

**Figure 9:** The effect of DNA amount and electroporation parameters on transfection of RAMOS RA-1 cells with pME18SE GFP.

**Figure 10:** Comparison of RAMOS RA-1 cells transfected with pME18sEGFP, pME18sasFP499 or mock transfected.

**Figure 11:** (A): Quantum Simply Cellular Beads stained with mouse anti-human IgM-Alexa 488. (B) RAMOS cells stained with mouse anti-human IgM-Alexa 488. (C) Quantitation of IgM molecules on the surface of RAMOS RA-1 cells.

5 **Figure 12:** Comparison of IgM expression on RAMOS RA-1 cells of different passage number. (A) RAMOS RA-1 passage 1 (B) RAMOS RA-1 passage 14.

**Figure 13:** IgM expression on RAMOS RA-1 samples from different sources.

10 **Figure 14:** Schematic representation of sequential overlap extension PCR.

**Figure 15:** Schematic representation of mammalian expression vector pME18sCD26asfp499.

15 **Figure 16:** Comparison of expression of asFP499 with and without the CD26 anchor in RAMOS RA-1.

**Figure 17:** Comparison of expression of asFP499 with and without the CD26 anchor in HEK 293 T.

20

#### Key to the Sequence Listing

SEQ ID NO:1 - Heavy chain locus of Ramos RA-1 cells.

SEQ ID NO:2 - Sequence of promoter region of a VH4 allele.

25 SEQ ID NO:3 - Sequence of promoter region of a VH4 allele.

SEQ ID NO:4 - Sequence of promoter region of a VH4 allele.

SEQ ID NO:5 - Sequence of promoter region of a VH4 allele.

SEQ ID NO:6 - Sequence of promoter region of a VH4 allele.

SEQ ID NO:7 - Consensus sequence of SEQ ID NO's 2 to 6.

30 SEQ ID NO:8 - Plasmid pME18SasFP499.

SEQ ID NO:9 - Sequence of enhancer of human immunoglobulin D segment locus.

SEQ ID NO:10 - Sequence of enhancer of human immunoglobulin heavy locus on chromosome 14.

SEQ ID NO:11 - Sequence of sheep immunoglobulin heavy chain 5' intronic enhancer.

35 SEQ ID NO:12 - Sequence of mouse 3' IgH regulatory enhancer.

SEQ ID NO:13 - Sequence of murine IgH enhancer.



- SEQ ID NO:14 - Sequence of mouse 3' kappa enhancer.  
 SEQ ID NO:15 - Promoter sequence of mouse immunoglobulin VH gene.  
 SEQ ID NO:16 - Promoter sequence of mouse immunoglobulin V1 gene.  
 SEQ ID NO:17 - Promoter sequence of mouse immunoglobulin mu heavy chain gene.  
 5 SEQ ID NO:18 - Promoter sequence of mouse immunoglobulin VH gene.  
 SEQ ID NO:19 - Homo sapiens germline IgH chain (ProV4-39) gene fragment.  
 SEQ ID NO:20 - Homo sapiens germline IgH chain (ProV3-30) gene fragment.  
 SEQ ID NO:21 - Homo sapiens germline IgH chain (ProV3-9) gene fragment.  
 SEQ ID NO:22 - Homo sapiens germline IgH chain (ProV1-18) gene fragment.  
 10 SEQ ID NO:23 - GPI signal from human decay-accelerating factor.  
 SEQ ID NO:24 - Polynucleotide encoding SEQ ID NO:23.  
 SEQ ID NO:25 - GPI signal from porcine membrane dipeptidase.  
 SEQ ID NO:26 - Polynucleotide encoding SEQ ID NO:25.  
 SEQ ID NO:27 - GPI signal from rat ceruloplasmin.  
 15 SEQ ID NO:28 - Polynucleotide encoding SEQ ID NO:27.  
 SEQ ID NO:29 - GPI signal from mouse Thy-1.  
 SEQ ID NO:30 - Polynucleotide encoding SEQ ID NO:29.  
 SEQ ID NO:31 - Transmembrane domain of murine B7-1.  
 SEQ ID NO:32 - Polynucleotide encoding SEQ ID NO:31.  
 20 SEQ ID NO:33 - Signal sequence of CD59.  
 SEQ ID NO:34 - Polynucleotide encoding SEQ ID NO:33.  
 SEQ ID NO's 35 to 86, 88 to 90, 92 to 103, 105, 106, 108 to 120 - Oligonucleotide primers.  
 SEQ ID NO:87 - Plasmid 3kb15a-7-4T.  
 25 SEQ ID NO:91 - Plasmid KW2.  
 SEQ ID NO:104 - Plasmid pME18sCD26asFP499.  
 SEQ ID NO:107 - Sequence of coding region of AICDA cDNA.  
 SEQ ID NO:110 - Plasmid KW3.

### 30 Detailed Description of the Invention

#### General Techniques

The present invention is performed without undue experimentation using, unless  
 35 otherwise indicated, conventional techniques of molecular biology, microbiology,  
 virology, recombinant DNA technology, peptide synthesis in solution, solid phase

peptide synthesis, and immunology. Such procedures are described, for example, in the following texts that are incorporated by reference:

1. Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, New York, Second Edition (1989), whole of Vols I, II, and III;
2. *DNA Cloning: A Practical Approach*, Vols. I and II (D. N. Glover, ed., 1985), IRL Press, Oxford, whole of text;
3. *Oligonucleotide Synthesis: A Practical Approach* (M. J. Gait, ed., 1984) IRL Press, Oxford, whole of text, and particularly the papers therein by Gait, pp1-22; Atkinson *et al.*, pp35-81; Sproat *et al.*, pp 83-115; and Wu *et al.*, pp 135-151;
4. *Nucleic Acid Hybridization: A Practical Approach* (B. D. Hames & S. J. Higgins, eds., 1985) IRL Press, Oxford, whole of text;
5. *Animal Cell Culture: Practical Approach*, Third Edition (John R.W. Masters, ed., 2000), ISBN 0199637970, whole of text;
6. *Immobilized Cells and Enzymes: A Practical Approach* (1986) IRL Press, Oxford, whole of text;
7. Perbal, B., *A Practical Guide to Molecular Cloning* (1984);
8. *Methods In Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.), whole of series;
9. J.F. Ramalho Ortigão, "The Chemistry of Peptide Synthesis" *In: Knowledge database of Access to Virtual Laboratory website* (Interactiva, Germany);
10. Sakakibara, D., Teichman, J., Lien, E. Land Fenichel, R.L. (1976). *Biochem. Biophys. Res. Commun.* **73** 336-342
11. Merrifield, R.B. (1963). *J. Am. Chem. Soc.* **85**, 2149-2154.
12. Barany, G. and Merrifield, R.B. (1979) in *The Peptides* (Gross, E. and Meienhofer, J. eds.), vol. 2, pp. 1-284, Academic Press, New York.
13. Wünsch, E., ed. (1974) *Synthese von Peptiden in Houben-Weyls Methoden der Organischen Chemie* (Müller, E., ed.), vol. 15, 4th edn., Parts 1 and 2, Thieme, Stuttgart.
14. Bodanszky, M. (1984) *Principles of Peptide Synthesis*, Springer-Verlag, Heidelberg.

15. Bodanszky, M. & Bodanszky, A. (1984) *The Practice of Peptide Synthesis*, Springer-Verlag, Heidelberg.
16. Bodanszky, M. (1985) *Int. J. Peptide Protein Res.* **25**, 449-474.

## 5 Hypermutating cells

In the context of the present invention, the hypermutating cells may be bacterial, yeast, avian, fungal, insect or mammalian cells. Examples of suitable hypermutating cells are described below.

10

### *Bacterial hypermutating strains :*

- (i) *Episcurian coli* mutator strain XL1-Red (triple DNA repair deficient – mutD, mutS, mut T) by Stratagene;
- (ii) *Escherichia coli* mutator strain MutD5 (MutD5-FIT, mutated DNAQ gene) Irving RA, Kortt AA, Hudson PJ (1996). *Immunotechnology* 2(2) 127-43;
- 15 (iii) *Escherichia coli* strain FC40. Foster PL (2000) *Bioessays* 22(12) : 1067-74 and Powell SC & Wartwell RM (2001) *Mutation Research* 473 (2) 219-28;
- (iv) Serogroup B meningococcal strains BF18, BF21 (defect in methyl – directed mismatch repair – lack DNA adenine methyltransferase (Dam) activity). Bucci *et al.* (1999) *Mol Cell* 3 (4) 435-45.
- 20

### *Yeast hypermutating strains:*

- (i) *Saccharomyces cerevisiae* strain (*mlh1*  $\Delta$  mutant). Shcherbakova & Kunkel (1999). *Molecular and Cellular Biology* 19(4) 3177-3183.
- 25 (ii) *Saccharomyces cerevisiae* strain DAG60 (*msh2* mutant). (deficient in mismatch repair system). Drotschmann *et al.* (1999) *Proc. Natl Acad Sci USA* 96 2970-2975.

### *Mammalian hypermutating strains:*

- 30 (i) Human B cell lines from Burkitt's lymphoma strains : RAMOS, BL2, BL41, BL70, Nalm-6. Sale & Neuberger (1998) *Immunity* 9 (6) 859-869.
- (ii) BL2 : Denepoux et al. (1997) *Immunity* 6(1) 35-46 and Poltorasky *et al.* (2001) *Proc Natl Acad Sci USA* 98 (14) 7976-81
- (iii) Human pre B cell line strain 18.81. Bachl *et al.* (2001) *Journal Immunology* 166 (8) 5051-7.
- 35

Intronic Enhancers

Examples of suitable exogenous intronic enhancers for use in the present invention include the following:

5

1) 1) DNA sequence of the human immunoglobulin D segment locus (Rabbitts et al (1983) Nature 306 (5945), 806-809):

5' CGGCCCCGATGCGGGACTGCGTTTTGACCATCATAAATCAAGTTTATTTT  
10 TTTAATTAATTGAGCGAAGCTGGAAGCAGATGATGAATTAGAGTCAAGATG  
GCTGCATGGGGGTCTCCGGCACCCACAGCAGGTGGCAGGAAGCAGGTCAC  
CGCGAGAGTCTATTTTAGGAAGCAAAAAAACACAATTGGTAAATTTATCAC  
TTCTGGTTGTGAAGAGGTGGTTTTGCCCAGGCCAGATCTGAAAGTGCTCT  
ACTGAGCAAAACAACACCTGGACAATTTGCGTTTCTAAAATAAGGCGAGGC  
15 TGACCGAAACTGAAAAGGCTTTTTTTAACTATCTGAATTTCAATTTCCAATCT  
TAGCTTATCAACTGCTAGTTTGTGCAAACAGCATATCAACTTCTAAACTGCA  
TTCATTTTTTAAAGTAAGATGTTTAAGAAATTAAACAGTCTTAGGGAGAGTTT  
ATGACTGTATTCAAAAAGTTTTTTAAATTAGCTTGTTATCCCTTCATGTGTA  
ATTAATCTCAAATACTTTTTTCGATACCTCAGAGCATTATTTTCATAATGACT  
20 GTGTTTACAATCTTTTT 3' (SEQ ID NO:9)

2) Homo sapiens immunoglobulin heavy locus (IGH.1@) on chromosome 14 (Ravetch et al (1981) Cell 27 (3 Pt 2), 583-591):

25 5'GGCCCCGATGCGGGACTGCGTTTTGACCATCATAAATCAAGTTTATTTTTT  
TAATTAATTGAGCGAAGCTGGAAGCAGATGATGAATTAGAGTCAAGATGG  
CTGCATGGGGGTCTCCGGCACCCACAGCAGGTGGCAGGAAGCAGGTCACC  
GCGAGAGTCTATTTTAGGAAGCAAAAAAACACAATTGGTAAATTTATCACT  
TCTGGTTGTGAAGAGGTGGTTTTGCCCAGGCCAGATCTGAAAGTGCTCTA  
30 CTGAGCAAAACAACACCTGGACAATTTGCGTTTCTAAAATAAGGCGAGGCT  
GACCGAAACTGAAAAGGCTTTTTTTAACTATCTGAATTTCAATTTCCAATCTT  
AGCTTATCAACTGCTAGTTTGTGCAAACAGCATATCAACTTCTAAACTGCAT  
TCATTTTTTAAAGTAAGATGTTTAAGAAATTAAACAGTCTTAGGGAGAGTTT  
ATGACTGTATTCAAAAAGTTTTTTAAATTAGCTTGTTATCCCTTCATGTGAT  
35 AATTAATCTCAAATACTTTTTTCGATACCTCAGAGCATTATTTTCATAATGAC  
TGTGTTTACAATCTTTTT 3' (SEQ ID NO:10)

3) *Ovis aries* immunoglobulin heavy chain 5' intronic enhancer (Dufour et al (1996) J. Immunol 156:2163-2170):

5 5'CTGCGAATACCGAGACGGGGCCTCTCAAAGCCACCCCTGATAGTCTGGAA  
AATTGAACTTTAAAAAGAGAGATGTTTAAAGTATTTTAAATTTTATCATT  
TAATTAACAACCTGCGAATCATGGCTTTGGAGAGTTGAGTAAGAGTTTGGCT  
GAAAAGTACTAACTAGGTTCCATCGGCCCTCGGCCCAATTCAGGGCTGTT  
TTGAGAATAATAAATTCAGCTTATTTTTTTAATGTAATTGGTGGTGCCGAGT  
10 TAGTCAAGATGGCCACGGGCCAGACTGACCACCTGCAGCAGGTGGCAGGA  
AGCATGTCCACTTGAGAGTCTGTTTTTGGAAAGCAAGAAAAAACAGTTGGTA  
AATTTATCGCTTCTGGTTTCCAAAAGGTGGTTTGCGGCTGGTTTTGCCCAGC  
CCCACAGAACCGAAAGTGTTCCACTGAGCACAACAGCACCTGGCTAATTTG  
CATTTCTAAAATAAGGCGCAGATGCTGACCGAAACTGGAAGGTTCTCTTC  
15 TAACTATTTGAGTTAACTTCAGCTTTAGCTTATCAACTGCTCACTTATCTTCA  
TTTTCAAAGTCGATGTTTAAGAAAGCCACCTGTCTCGGGTGCACTGTCTCGG  
TGCATTGCTGCACTCTCTGATGAGCCGTCCTTCAAGGTGGTTGAGCTGAG 3'  
(SEQ ID NO:11)

20 4) Mouse 3' IgH regulatory enhancers (C alpha3'E and hs3) (Saleque et al (1997) J. Immunol. 158 (10), 4780-4787):

5'CTAGATACTGAGTTCTGGTTCTAATAACTGGCTCCTGTACTGATGGATGG  
GTCCTGACTAGTCATTGGGGCCCTGATCCTCAACATTGACTTCAAAACCTGAA  
25 CTCTAGCCCCATGCCTCATTACATTAGGATGATCCCTACAGGGGATTCCTG  
CAGAAGATTCCAGAATCCCCACAACACTGTTACACACTGGGCTGCAACTG  
GGACAGTGACCCTTTTGACTCATAGGACTTGCCAGGCACAGAGGCACAGAA  
TGGAGACAAAGCAAGCCCAGGACCCTGGAGATGGAGCCTCTGGTGGGGTC  
TACAGATGTGGGGTCAGCATCGTAGGGAGGTTTGCAGGGCAGGTGTGGGG  
30 CAGGGCAGAGGTAGTCATGCTTATAGATACTATTTTTCTCTCCTCTGGAGCC  
TCCTTTGTCTATCACCTGCTGTCCTGGGATCTCTATCTGGGGTCAACAATGT  
TTGCAGTACAGGTGTGGGGGTAGGGCAGGGATGCTCACATTAGCAACTTGT

TTTTCTCTCTTCTGAAGTCTCTGTTGTCTATCACCTGCTGAAACATTCAAAGC  
AGCTCTCAGCTGAGGGCAGCTGAGTCATCCTGAGCCTGTCTCAGCACAGGT  
GCCCCAAACCAGAGCTACTGTTCTGAGAATCACATCACACTGGACCAGGCC  
AGGTGGGCCTGGGACATGGATGAGGGGTGGGAGCCAGGGGAGCCTGCCAG  
5 GGGCTGAGGAGGCCCCAACCCCCACTACCCAAGGCCATCCACACCTGTGCC  
TTAGTGAGGCCATGTTCTGTCCCAATGAGAACAAGTCCAATTAAGATTAAG  
TATGGTCTTCCCAGGACTATCCAGAGCTAAGGGGTGTCAGCCAGGGACAAC  
CCAGACCAGCCTGAGGTCAGCCAGCATCACCCAAGGCCACACAGCTATTCT  
GGCTAGAGGACTAGATAGCTAGCTCATCGAGGCCCTGGAGATGCAGAATG  
10 GAAGAGTTTATCCCTGCCAGACAGGGCTCATCAGAAAGGCAGGTATCTCAC  
TACACATGACCTCCCTGAATATTTCCCAGAGTCCAGTTGGTTCTAG 3' (SEQ  
ID NO:12)

5) Murine IgH enhancer (Kadesch et al (1986) Nucleic Acids Res. 14 (20), 8209-  
15 8221):

5'AGTCAAGATGGCCGATCAGAACCAGAACACCTGCAGCAGCTGGCAGGAA  
GCAGGTCATGTGGCAAGGCTATTTGGGGAAGGGAAAATAAAACCACTAGG  
TAAACTTGTAGCTGTGGTTTGAAGAAGTGGTTTTGAAACACTCTGTCCAGCC  
20 CCACCAAACCGAAAGTCCAGGCTGAGCAAAACACCACCTGGGTAATTTG  
CATTTCTAAA ATAAGTTGAGG 3' (SEQ ID NO:13)

### 3' kappa enhancers

25 An example of a suitable exogenous 3' kappa enhancer for use in the present invention  
is as follows.

1) Murine 3' kappa enhancer (Meyer and Neuberger (1989) EMBO J. 8 (7), 1959-  
1964):

30

5'AGCTCAAACCAGCTTAGGCTACACAGAGAACTATCTAAAAAATAATTA  
CTAACTACTTAATAGGAGATTGGATGTTAAGATCTGGTCACTAAGAGGCAG  
AATTGAGATTCTGAACCAGTATTTTCTACCTGGTATGTTTTAAATTGCAGTAA

GGATCTAAGTGTAGATATATAATAATAAGATTCTATTGATCTCTGCAACAA  
CAGAGAGTGTTAGATTTGTTTGGAAAAAATATTATCAGCCAACATCTTCT  
ACCATTTTCAGTATAGCACAGAGTACCCACCCATATCTCCCCACCCATCCCC  
ATACCAGACTGGTTATTGATTTTCATGGTGACTGGCCTGAGAAGATTAAAA  
5 AAAGTAATGCTACCTTATTGGGAGTGTCCCATGGACCAAGATAGCAACTGT  
CATAGCTACCGTCACACTGCTTTGATCAAGAAGACCCTTTGAGGAACTGAA  
AACAGAACCTTAGGCACATCTGTTGCTTTTCGCTCCCATCCTCCTCCAACAGC  
CTGGGTGGTGCACTCCACACCCTTTCAAGTTTCCAAAGCCTCATACACCTGC  
TCCCTACCCCAGCACCTGGCCAAGGCTGTATCCAGCACTGGGATGAAAATG  
10 ATACCCACCTCCATCTTGTTTGATATTACTCTATCTCAAGCCCCAGGTTAG  
TCCCCAGTCCCAATGCTTTTGCACAGTCAAACTCAACTTGGAATAATCAGT  
ATCCTTGAAGAGTTCTGATATGGTCACTGGGCCCATATACCATGTAAGACA  
TGTGGAAAAGATGTTTCATGGGGCCCAGACACGTTCTAG 3' (SEQ ID NO:14)

15 Promoter sequences

Examples of suitable exogenous promoter sequences for use in the present invention include:

20 1) (Kataoka et al (1982). J Biol. Chem. 257 : 2777-285):

5' AAGCAGCCCTCAGGCAGAGGATAAAAGCTCACACTAACTGAGAAGC  
TCCATCCTCTTCTC 3' (SEQ ID NO:15)

25 2) (Clarke et al (1982). Nucleic Acids Res. 10 : 7731):

5' AATTAGGCCACCCTCATCACATGAAAACCAGCCCAGAGTGACTCTAG  
CAGTGGGATCCTG 3' (SEQ ID NO:16)

30 3) Grosschedl and Baltimore D., (1985). Cell 41: 885-897):

5' CATGTGCGACTGTGATGATTAATATAGGGATATCCACACCAAACATC  
ATATGAGCCCTAT 3' (SEQ ID NO:17)

35 4) (Schiff et al (1986). J. Exp. Med. 163 : 573-587):

5' AACATGAGTCTGTGATTATAAATACAGAGATATCCATACCAAACAAC  
TTATGAGCACTGT 3' (SEQ ID NO:18)

5 Murine B lymphocyte VH promoters (eg. V1 Ig VH promoter , BCL1 VH promoter)  
may also be used in the methods of the present invention.

Human Immunoglobulin heavy chain promoters

10 The following human heavy chain promoters are described in in Haino et al (1994) J.  
Biol. Chem. 269 (4), 2619-2626:

1) Homo sapiens germline IGH chain (ProV4-39) gene fragment:

15 5'ATCCCAAAATCTGTCNTTGATCCAGGATCACACTCATCTCTCAGACCAGC  
TCCTTCAGCACATCTCTTTACCTGGAAGAAGAGGACTCTGGGCTTGGAGAG  
GGGAGCCCCCAAGAAGAGAACTGAGTTCTCAAAGGGCACAGCCAGCATTC  
TCCTCCCAGGGTGAGCTCAAAGACTGGCGCCTCTCTCATCCCTTTTCACTG  
CTCCGTACAAACGCACCAACCCCATGCAAATCCTCACTTAGGCGCCACAG  
20 GAAGCCACCACACATTTCTTAAATTCAGGTCCAACATCATAAGGGAAATGC  
TTTCTGAGAGTCATGGATCTCATGTGCAAGAAA 3' (SEQ ID NO:19)

2) Homo sapiens germline IGH chain (ProV3-30) gene fragment:

25 5'AGATATAACTATATTTTCCTGAATGATGGAATTACTACCAGTCTCCCCCA  
GGACACTTCATCTGCCCTGAGCCCAGCCTCTCCTCAGATGTCCCACCCAGA  
GCTTGCTATATAGTGGGGGACATGCAAATAGGGCCCTCCCTCTACTGATGA  
AAACCAGCCCAGCCCTGACCCTGCAGCTCTGGGAGAGGAGCCCAGCACTA  
GAAGTCGGCGGTGTTTCCATTCGGTGATCAGCACTGAACACAGAGGAC  
30 TCACC 3' (SEQ ID NO:20)

3) Homo sapiens germline IGH chain (ProV3-9) gene fragment:

35 5'CAGTAGAAATGCTAATAAGAATTAATTGTTTATGAAGTGTAATCACTCTG  
GGACACAGCCCCTCAGAGGCATCCCTTCCAGAACCCGCTATATAGTAGGA  
GACATGCAAATAGGGCCCTCCCTCTGCTGATGAAAACCAGCCCAGCCCTGA



CCCTGCAGCTCTGGGAGAGGAGCCCCAGCCCTGAGATTCCCAGGTGTT  
TCCATTCAGTGATCAGCACTGAACACAGAGGACTCACC 3' (SEQ ID NO:21)

4) Homo sapiens germline IGH chain (ProV1-18) gene fragment:

5

5'GATGGGTAGGGGATGCGTGTCTCTAACAGGATTACGTCTTGAACCCTCA  
GCTTCTACAATTGTGTCGTCCATGTGTCATGTATTTGCTCTTTCTCATCCTGG  
GTCAGGAATTGGGCTATTAAATAGCATCCTTCATGAATATGCAAATAACTG  
AGGTGAATATAGATATCTGTGTGCCCTGAGAGCATCACCCAAAAACCACAC  
10 CCCTCCTTGGGAGAATCCCCTAGATCACAGCTCCTCACC 3' (SEQ ID NO:22)

Methods for selection of nucleic acids or proteins/peptides with an altered phenotype

15 The terms "altered phenotype", "desired activity" and "altered activity" are generally used interchangeably herein.

In particular embodiments of the present invention, the mutated nucleic acid, or gene product encoded thereby, is subjected to an assay for identifying an altered phenotype. Suitable procedures for identifying altered phenotypes include, but are not limited to, those described below.

20

Protein/peptide display and selection

25 In one embodiment of the invention, proteins encoded by nucleic acids obtained using the methods of the invention are displayed on the surface of the hypermutating cells.

One well-known peptide display method involves the presentation of a peptide sequence on the surface of a filamentous bacteriophage, typically as a fusion with a bacteriophage coat protein. The bacteriophage library can be incubated with an immobilized, predetermined macromolecule or small molecule (e.g., a receptor) so that bacteriophage particles which present a peptide sequence that binds to the immobilized macromolecule can be differentially partitioned from those that do not present peptide sequences that bind to the predetermined macromolecule. The bacteriophage particles (i.e., library members) which are bound to the immobilized macromolecule are then recovered and replicated to amplify the selected bacteriophage subpopulation for a subsequent round of affinity enrichment and phage replication. After several rounds of

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affinity enrichment and phage replication, the bacteriophage library members that are thus selected are isolated and the nucleotide sequence encoding the displayed peptide sequence is determined, thereby identifying the sequence(s) of peptides that bind to the predetermined macromolecule (e.g., receptor). Such methods are further described in  
5 WO 91/17271, WO 91/18980, WO 91/19818 and WO 93/08278.

WO 93/08278 describes a recombinant DNA method for the display of peptide ligands that involves the production of a library of fusion proteins with each fusion protein composed of a first polypeptide portion, typically comprising a variable sequence, that  
10 is available for potential binding to a predetermined macromolecule, and a second polypeptide portion that binds to DNA, such as the DNA vector encoding the individual fusion protein. When transformed host cells are cultured under conditions that allow for expression of the fusion protein, the fusion protein binds to the DNA vector encoding it. Upon lysis of the host cell, the fusion protein/vector DNA  
15 complexes can be screened against a predetermined macromolecule in much the same way as bacteriophage particles are screened in the phage-based display system, with the replication and sequencing of the DNA vectors in the selected fusion protein/vector DNA complexes serving as the basis for identification of the selected library peptide sequence(s).

20 The displayed protein/peptide sequences can be of varying lengths, typically from 3-5000 amino acids long or longer, frequently from 5-100 amino acids long, and often from about 8-15 amino acids long. A library can comprise library members having varying lengths of displayed peptide sequence, or may comprise library members  
25 having a fixed length of displayed peptide sequence. Portions or all of the displayed peptide sequence(s) can be random, pseudorandom, defined set kernel, fixed, or the like. The display methods include methods for display of single-chain antibodies, such as nascent scFv on polysomes or scFv displayed on phage, which enable large-scale screening of scFv libraries having broad diversity of variable region sequences and  
30 binding specificities.

Another method of display is bacterial surface display. The protein of interest is expressed on the bacterial cell surface as a fusion with one of the following proteins, OmpA, LamB, PhoE, FliC, PALD, and EaeA intimin. Alternatively it may be  
35 expressed in the periplasm (periplasmic expression with cytometric screening, (PECS)) (Chen *et al.*, 2001). Whilst experiments demonstrate expression on the bacterial cell

surface, this display system is not operating as well or used as frequently as the yeast surface display. The nature of a prokaryotic system itself may explain why the system is not always preferred. Firstly, bacteria lack the protein folding and post-translational modification machinery required for the presentation of an eukaryotic protein.

5 Secondly, the protein of interest needs to be expressed in a soluble form. Thirdly, steric interference from the bacterial lipopolysaccharide layer can potentially impede binding to larger macromolecular antigens. Despite being a single cell system (one plasmid to each bacterium), capable of displaying thousands of copies of the protein of interest on the cell surface and being amenable to screening using flow cytometry, this  
10 system offers no additional advantages over the yeast surface display system.

A further method of display is yeast surface display. The protein of interest is fused to an alpha agglutinin subunit called Aga2p and expressed on the surface of the yeast cell. This form of display appears to be very successful and offers several advantages over  
15 other display systems. These include; correct protein folding and secretion (homologous to that in mammalian cells), display of large numbers of protein on yeast cell surface, single cell system (i.e. each yeast cell contains only one plasmid copy), system is amenable to screening using flow cytometry which offers finer quantitative discrimination between mutants and the dissociation constant ( $K_D$ ) can be estimated *in*  
20 *situ* in the display format without having to subclone. The major disadvantage of this system is that the library size is restricted due to low transfection efficiency. To date no one has managed to overcome this limitation of the system.

Proteins can be anchored to the surface of mammalian cells via a number of different  
25 anchors including, but not limited to, Type I transmembrane domains (TM I), type II transmembrane (TM II) and glycosylphosphatidylinositol (GPI).

Examples of suitable GPI anchor attachment signals include:

- 30 (i) GPI signal from human decay-accelerating factor (DAF). Caras IW, Weddell GN, Davits MA, Nussenzweig W, Martin DW. (1987) Science 238(4831):1280-3  
Accession No.: AY055758  
Peptide sequence: PNKGSTTSGTTRLLSGHTCFTLTGLLGTLVTMGLLT (SEQ ID NO:23)  
35 Nucleotide sequence: 1158-1268

CCAAATAAAGGAAGTGGAACCACTTCAGGTACTACCCGTCTTCTATCTGGG  
CACACGTGTTTCACGTTGACAGGTTTGCTTGGGACGCTAGTAACCATGGGC  
TTGCTGACT (SEQ ID NO:24)

- 5 (ii) GPI signal from porcine membrane dipeptidase. Hooper NM, Low MG, Turner AJ. (1987) Biochem. J. 244(2):465-9

Accession No.: E04233

Peptide sequence: CRTNYGYSAAPSLHLPPGSLLASLVPLLLSLP (SEQ ID NO:25)

- 10 Nucleotide sequence: 1248-1346

TGCCGGACGAATTACGGCTACTCAGCCGCCCCCAGCCTCCACCTCCCGCCG  
GGCTCGCTGCTGGCCTCCCTCGTGCCCCCTCCTCCTCCTCAGTCTTCCG (SEQ ID NO:26)

- 15 (iii) GPI signal from rat ceruloplasmin. Patel BN, Dunn RJ & David S. (2000) J. Biol. Chem. 275(6):4305-10

Accession No.: AF202115

Peptide sequence: ASSQSYRMTWNILYTLLISMTTLFQISTKE (SEQ ID NO:27)

Nucleotide sequence: 3161-3252

- 20 GCATCGTCTCAGAGCTACAGGATGACCTGGAACATACTCTATACTGTTA  
ATCAGCATGACTACTTTATTCCAAATATCTACCAAGGAG (SEQ ID NO:28)

- (iv) GPI signal from mouse Thy-1. Bernasconi E, Fasel N & Wittek R. (1996) J. Cell Sci. 109(6):1195-201

- 25 Peptide sequence:  
SSNKSISVYRDKLVKCGGISLLVQNTSWMLLLLLLSLSLLQALDFISL (SEQ ID NO:29)

Nucleotide sequence:

- 30 AGCTCCAATAAAAGTATCAGTGTGTATAGAGACAAGCTGGTCAAGTGTGGC  
GGCATAAGCCTGCTGGTTCAGAACACATCCTGGATGCTGCTGCTGCTTT  
CCCTCTCCCTCCTCCAAGCCCTGGACTTCATT (SEQ ID NO:30)

- (v) Dictyostelium discoideum protein 1I. Stevens BA, White IJ, Hames BD Hooper NM. (2001) Biochimica et Biophysica Acta 1511: 317-329.

(vi) *Plasmodium falciparum* merozoite surface protein-1. Burghas PA, Gerold P, Pan W, Schwartz RT, Lingelbach K, Burjard H. (1999) *Molecular and Biochemical Parasitology* 104:171-183.

5 An example of a suitable transmembrane domain for use as an anchor domain in the present invention is:

(i) The transmembrane domain of murine B7-1. Chou W., Liao K., Jiang S.Y., Yeh M.Y. and Roffler S.R.(1999) *Biotechnol Bioeng.* 1999 Oct 20;65(2):160-9.

10 Accession No.:AH00465S3

Peptide sequence: PEDPPDSKNTLVLFAGFGAVITVVVIVVIKCFCKH (SEQ ID NO:31)

Nucleotide sequence: 171-281:

15 CCCAGAAGACCCTCCTGATAGCAAGAACACACTTGTGCTCTTTGGGGCAGG  
ATTCGGCGCAGTAATAACAGTCGTCGTCATCGTTGTCATCATCAAATGCTTC  
TGTAAGCAC (SEQ ID NO:32)

Other suitable examples include those provided in Table 1.

20 **Table 1 : Examples of anchors suitable for surface display:**

Anchor type	Molecule name	Accession No.
Transmembrane type I (TM I)	CD1a	X04450 (gi 32495)
	CD68	BC05557(gi 33869409)
Transmembrane type II (TM II)	CD10	Y00811 (gi 29625)
	CD13	X13276 (gi 28677)
	CD26	M74777 (gi 180082)
Glycosylphosphatidylinositol (GPI)	CD14	X113334 (gi 29740)
	CD24	M58664 (gi 180167)
	CD48	M59904 (gi 180138)
	CDw52	X62466 (gi 29645)
	CD55	M31516 (gi 181467)
	CD59	X16447(gi 29805)
	CD67	X52378 (gi 29918)

### Signal peptide sequences

For TM1 and GPI anchors, an N-terminal signal sequence as well as a C-terminal signal sequence is preferably be added to the polypeptide if it is not normally found on the cell surface. For TM2 the signal and anchor sequence are one and the same and are added to the N-terminus of the polypeptide. To achieve adequate levels of surface expression, it may be necessary to mutate the initiation codon of the target gene so that only chimeric proteins consisting of the signal and/or anchor fused to the target protein are produced.

An example of an appropriate signal sequence is the signal sequence of CD59:

Accession No.:X16447 (gi 180082)

Peptide sequence: MGIQGGSVLFGLLVLAVFCHSGHS (SEQ ID NO:33)

Nucleotide sequence: 64-138

5'ATGGGAATCCAAGGAGGGTCTGTCCTGTTTCGGGCTGCTGCTCGTCCTGGC  
TGTCTTCTGCCATTCAGGTCATAGC 3' (SEQ ID NO:34)

Proteins/peptides encoded by mutant nucleic acids obtained using the methods of the invention can be used in a number of yeast based methods to detect protein-protein interactions. One well known system is the yeast two-hybrid system (Fields and Song 1989) which has been used to identify interacting proteins and to isolate the corresponding encoding genes. In this system, prototrophic selectable markers which allow positive growth selection are used as reporter genes to facilitate identification of protein-protein interactions. Related systems which may be employed include the yeast three-hybrid system (Licitra and Liu 1996) and the yeast reverse two-hybrid system (Vidal et al. 1996). Such procedures are known to those skilled in the art.

### Examples

#### Example 1: Defining a region in RAMOS cells for integration of target genes

#### *Methods & Materials*

##### *Cell line and cell culture conditions*

The RAMOS strain RA 1 was obtained from the American Tissue Culture Collection (ATCC-CLR-1596). This strain is IgM positive and expresses the interleukin 4 (IL-4) and CD23 receptors. Cells were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 10% heat inactivated fetal calf serum (FCS) and penicillin (100 U / ml) and streptomycin (100 µg / ml), and incubated at 37 °C with 5% CO<sub>2</sub>.

#### *Extraction of DNA from cells*

Cells were harvested and centrifuged at 1500 rpm and resuspended in PBS. DNA was extracted from cells using a Genoprep DNA isolation kit (Scientifix, Australia) according to manufacturer's instructions. Briefly, after removing the supernatant 375 µl of lysis and binding solution was added to cells ( $5 \times 10^5$ ), together with 20 µl Genoprep DNA magnetic beads. This mixture was vortexed for five seconds then incubated at room temperature for a minimum of ten minutes on a rocker, or rotating wheel. Beads with attached DNA were collected using a magnet, the supernatant was removed and 450 µl of washing solution was added. The mixture was subsequently vortexed for five seconds and the beads were collected as described previously. This washing procedure was repeated twice, with the final wash solution being 70 % ethanol. Beads were resuspended in 450 µl of 70 % ethanol and transferred to a new tube. After removing the supernatant, 450 µl of sterile water was added to the beads and removed immediately. The beads were then resuspended in 200 µl of sterile water and incubated at 70 °C for two minutes to elute the DNA. The beads were collected again using a magnet and the supernatant containing the eluted DNA was transferred to a new tube.

The quantity and quality of isolated DNA was determined by spectrometry and electrophoresis. A culture containing  $5 \times 10^5$  cells consistently yielded 10 ng / µl of DNA. This DNA migrated as a single band at approximately 23 kbp on a 0.9 % gel indicating that the genomic DNA was intact.

#### *Sequencing of the 5' region upstream of the rearranged VH allele*

The homologous sequence upstream of the site of integration chosen for the vector corresponds to a ~ 5kb fragment between the VH<sub>7-35</sub> and VH<sub>4-34</sub> alleles of the immunoglobulin heavy chain locus of the RAMOS cell line (corresponding to the sequence gi 4512287 nucleotides 54521 – 59517).

RAMOS RA-1 genomic DNA was prepared using the Genoprep DNA isolation kit (Scientifix, Australia). Platinum Pfx DNA polymerase (GibcoBRL, Life Technologies) was used to amplify fragments varying from 300bp to 1000 bp from genomic DNA. The reaction included 1 X Pfx amplification buffer, 50 mM  
5 Magnesium sulfate, 1 X PCR enhancer solution, forward primer (10 pM), reverse primer (10 pM), dNTPs (10 mM each) template DNA (100 ng), platinum Pfx DNA polymerase (0.6 U) and sterile water in a final volume of 20 µl. Cycling conditions were as follows; one cycle of 95°C for 5 minutes, 30 cycles of 95°C for seconds, 60°C for 30 seconds, 68°C for one and a half seconds, and one cycle of 72°C for 7 minutes.  
10 Annealing temperatures and extension times for some primer sets varied, ranging from 55°C to 65°C and one minute to two and a half minutes respectively. Primers were designed based on the human germline DNA for immunoglobulin heavy-chain variable region, complete sequence gi 4512287 nucleotides 54786 to 59721 (Table 2). A second PCR reaction was performed using 0.5 / 20 µl of the first PCR as a template to gain  
15 sufficient DNA for cloning.

PCR products were run on 1.0 % agarose gels and DNA was extracted using Nucleospin Extraction Kit (Nagel-Macherey, Germany) according to manufacturer's instructions. The purified DNA was digested with restriction enzymes EcoR I and Hind  
20 III. Digested products were cleaned up and concentrated using phenol extraction followed by ethanol precipitation. These products were ligated into into pBluescript SK + (Stratagene, Texas, USA) and transformed into *Escherichia coli* XL1 Blue electro-competent bacteria. Minipreps were prepared from 5 ml overnight cultures using QIAprep miniprep spin kit (Qiagen, CA, USA) and sequenced using an ABI 373 DNA  
25 sequencer with primers T3 and T7.

Sequences were analysed using BLAST program (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>) and assembled in Clone Manager Suite 7 (Scientific and Educational Software). The assembled sequence is set out as  
30 nucleotides 1 to 5190 of SEQ ID NO:1. This sequence shares 99 % similarity with the published sequence gi 4512287 nucleotides 54521–59517.

*Cloning of the 5kb fragment upstream of the rearranged VH allele from genomic DNA*

35 Genomic DNA was prepared using Genoprep DNA isolation kit (Scientifix, Australia). Platinum Pfx DNA polymerase (Invitrogen, CA. USA ) was used to amplify the 5 kb



fragment from genomic DNA. The reaction included 1 X Pfx amplification buffer, 50 mM magnesium sulfate, 1X PCR enhancer solution, forward primer 8771 (5'CCATCGATAATTTAGTTTTTCACGGGGCATCTGCAGGGT 3') 10 pM, reverse primer 8872 (5' GGGGTACCGTTCTTGTGCAGGAGGTCCATGACTCTCAG 3') 10 pM, dNTPs (10 mM each) template DNA (100 ng), platinum Pfx DNA polymerase (0.6 U) and sterile water in a final volume of 20 µl. Cycling conditions were as follows ; one cycle of 94 °C for 15 seconds, fifteen cycles of 94°C for 10 seconds, 68°C for 3.5 minutes, 15 cycles of 94°C for 10 seconds, 68°C for 3.5 minutes with and an extra 15 seconds added each cycle, and one cycle of 72°C for 7 minutes. A second PCR reaction was performed using 0.5 / 20.0 µl of the first PCR reaction as a template to gain sufficient DNA for detection using ethidium bromide.

PCR products were run on a 0.9 % agarose gel and DNA was extracted using Gel Extraction Kit (Qiagen, CA, USA). Purified DNA was cloned into pPCRScript using the PCR-Script Amp cloning kit (Stratagene, Texas, USA) at the Srf I site. The resulting construct was referred to as 5kbPCRScript 15a-7 (7971 bp). This construct was sequenced using primers in Table 3.

**Table 2: Primers used for genomic PCR to sequence the 5' region upstream of the rearranged VH allele (VH<sub>4-34</sub>) in RAMOS RA-1**

Primer Name	Sequence	Homologous sequence in gi AB019439
8444	5' CCGGAATTCAATTTGAGATTGTGTGTGAGATCTCAGGAG 3' (SEQ ID NO:35)	NT 58890 - 58920
8436	5' CCGGAATTC ATAGACAGCGCAGGTGAGGGACAG GGTCTC 3' (SEQ ID NO:36)	NT 59702 - 59731
8438	5' CCGGAATTCCTG AGA ACTCAG TTCTCTTCCTGTGGCCTC 3' (SEQ ID NO:37)	NT 59281 - 59310
8440	5' CCGGAATTCAATTTGAGATTGTGTGTGAGATCTCAGGAG 3' (SEQ ID NO:38)	NT 58890 - 58920
8441	5' CCCAAGCTTTCCTGTTACAACATCCATGGAGATATTTTG 3' (SEQ ID NO:39)	NT 58420 - 58450
8442	5' CCGGAATTC TGAATTGCAAGAACATACCCTAGGGTGTGC 3' (SEQ ID NO:40)	NT 58500 - 58530

8464	5' CCGGAATTCTAGGGCAAACAGAGGCCAGATGTTTGAGGAG 3' (SEQ ID NO:41)	NT 57720 - 57750
8466	5' CCGGAATTCAATTTAACAGCATAAAAACGATCAGTCCAA 3' (SEQ ID NO:42)	NT 57330 - 57360
8470	5' CCGGAATTCCGTGTTTCTGGAGCAGGGCATGGCTTTGGG 3' (SEQ ID NO:43)	NT 56550 - 56580
8472	5' CCGGAATTCGTTGGGTTCCCAGTG TAGGTGATGATCCAT 3' (SEQ ID NO:44)	NT 56160 - 56190
8550	5' CCGGAATTCTCCCAGGAAGTGGGTTATTTTAAATAGTA 3' (SEQ ID NO:45)	NT 58951- 58981
8553	5' CCGGAATTCACTATAGTCACCTCAGTTAATTGCATATTC 3' (SEQ ID NO:46)	NT 55770 - 55800
8554	5' CCCAAGCTTGACTTCCTTTAAAAATATCTAAAATAAGTA 3' (SEQ ID NO:47)	NT 55300 - 55330
8555	5' CCGGAATTCGGTTCTCATTACAACATCCAGTTTGATAAA 3' (SEQ ID NO:48)	NT 55380 - 55410
8557	5' CCGGAATTCCTCCAAGAAAAGATCTCATGCATCACCAGG 3' (SEQ ID NO:49)	NT 54990 - 55020
8558	5' CCCAAGCTTAATTTAGTTTTACGGGGCATCTGCAGGGT 3' (SEQ ID NO:50)	NT 54520 - 54550
8606	5' CCCAAGCTTTGCACACCCTAGGGTATGTTCTTGCAATTC 3' (SEQ ID NO:51)	NT 58500 - 58530
8607	5' CCCAAGCTTCCCAAAGCCATGCCCTGCTCCAGAAACACG 3' (SEQ ID NO:52)	NT 56550 - 56580
8608	5' CCGGAATTC CCATAATATGTGAATGCGTTATTTAGG GAA 3' (SEQ ID NO:53)	NT 55500 - 55529
8687	5' CCCAAGCTTCATGTTCCACGCATTACGTC 3' (SEQ ID NO:54)	NT 54336 - 54355
8689	5' CCCAAGCTTAAGAGTGTTTGGGTCACCG 3' (SEQ ID NO:55)	NT 54927 - 54946

**Table 3 : Primers used for sequencing clones containing the 5 kb region upstream of the rearranged VH allele (VH<sub>4-34</sub>) in RAMOS RA-1**

Primer Name	Sequence	Homologous sequence in gi AB019439
8445	5' CCCAAGCTTTTAACTCAGGAGGACTCAATACACCCTGGA 3' (SEQ ID NO:56)	NT 57640-57670
8443	5' CCCAAGCTTAAACAATACCTACAAATTCAGAAGCTCTTT 3' (SEQ ID NO:57)	NT 58030-58060
8471	5' CCCAAGCTT AAGTCTTCTGGTTACACCTTCACCAT TAT 3' (SEQ ID NO:58)	NT 56080-56110
8465	5'CCCAAGCTTACTCTCTTCCCTCTGTGACTAGAGCTCTGT 3' (SEQ ID NO:59)	NT 57250-57280
8473	5' CCCAAGCTTTCAGCTTCTACAGTTGTGTACCCCATGTGT 3' (SEQ ID NO:60)	NT 55690-55720
8609	5' CCCAAGCTTTGCAGAGTTCACTGGGTTTCCTAAAGG CAA 3' (SEQ ID NO:61)	NT 55027-55056
8467	5' CCCAAGCTTTCACCACAAAGGAACTTTCATCTCTCCTGG 3' (SEQ ID NO:62)	NT 56860-56890
8439	5' CCCAAGCTTTTTTACACAGAAATGTTTAGAGGT CAGGCC 3' (SEQ ID NO:63)	NT 58810-58840
8606	5' CCCAAGCTTTGCACACCCTAGGGTATGTTCTTGCAATTC 3' (SEQ ID NO:64)	NT 58500- 58530
8551	5' CCCAAGCTTCCCAAAGCCATGCCCTGCTCCAGAAACACG 3' (SEQ ID NO:65)	NT 56550- 56580
0003	5' CCCAAGCTTATGATGTAACCCTCATTGGCCTCA 3' (SEQ ID NO:66)	NT 54497-54520
0006	5' CCGGAATTCGAGACTCCAAGAAAAGATCTCATG 3' (SEQ ID NO:67)	NT 55001-55024
0007	5' CCCAAGCTTATGTTCTTGCAATTCAGCGGAGGA 3' (SEQ ID NO:68)	NT 58514-58537
0008	5' CCGGAATTCTGTGTGAGATCTCAGGAGAAGGTA 3' (SEQ ID NO:69)	NT 58885-58908

5 *PCR amplification of rearranged VH, D and JH segments from genomic DNA*

Genomic DNA was prepared using the Genoprep DNA isolation kit (Scientifix, Australia). Platinum Pfx DNA polymerase (Invitrogen, CA, USA) was used to amplify the rearranged VH, D and JH genes from genomic DNA. The PCR reaction included 1X Pfx amplification buffer, 50 mM magnesium sulfate, 1X PCR enhancer solution, forward primer (10 pM), reverse primer (10 pM), dNTPs (10 mM each), template (100 ng), platinum Pfx DNA polymerase (0.6 U) and sterile water in a final volume of 20  $\mu$ l. Cycling conditions were as follows; one cycle of 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 65°C for 30 seconds, 68°C for 2.5 minutes, and one cycle of 72°C for 7 minutes. Primers used were specific for each of the seven VH family leader sequences together with a previously described consensus JH primer, JOL48, that anneals to all six human JH segments (Table 4). A second PCR reaction was performed using 0.5 / 20  $\mu$ l of the first PCR as template to gain sufficient DNA for detection using ethidium bromide.

PCR products were run on 1.5 % agarose gels and DNA was extracted from bands using Nucleospin Extraction Kit (Nagel-Macherey, Germany) according to manufacturer's instructions. A third PCR was performed on the purified DNA as described above. Products were cloned into pBluescript SK + (Stratagene, Texas, USA) using EcoR I and Hind III sites and sequenced as previously described.

**Table 4 : Primers for PCR amplification of rearranged VH, D and JH segments from genomic DNA.**

Primer Name	Sequence	Specificity
8111	5'CCCAAGCTTATGGACTGGACCTGGAGGATCCTCTTCTTGGTGGCAGCA 3' (SEQ ID NO:113)	VH1 leader
8116	5'CCCAAGCTTATGGACACACTTTGCTCCACGCTCCTGCTGCTGACC ATCCCT 3' (SEQ ID NO:114)	VH2 leader
8112	5'CCCAAGCTTATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTGCTATT 3' (SEQ ID NO:115)	VH3 leader
8113	5'CCCAAGCTTATGAAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCT 3' (SEQ ID NO:116)	VH4 leader
8118	5'CCCAAGCTTATGGGGTCAACCGCCATCCTCGCCCTCCTCCTGGCTGTTCTC 3' (SEQ ID NO:117)	VH5 leader
8114	5'CCCAAGCTTATGTCTGTCTCCTTCCTCATCTTCCTGCCCCTGCTGGGCCTC 3' (SEQ ID NO:118)	VH6 leader
8115	5'CCCAAGCTTATGGACTGGACCTGGAGGATCCTCTTCTTGGTGGCAGCAGCA 3' (SEQ ID NO:119)	H7 leader
N8336 (JOL48)	5'CCCAAGCTTCCCCAAGCTTCCCAGGTGCAGCTACAGCAG 3' (SEQ ID NO:120)	Consensus JH

**Table 5 : Primer sequences used for genomic PCR to sequence the 3 kb region downstream of the rearranged VH allele (VH<sub>4-34</sub>) in RAMOS RA-1**

Primer Name	Sequence	Homologous sequence in NCBI database
8604	5' CCCAAGCTTCGGCCCCGATGCGGGACTGCGTTTTGACCA 3' (SEQ ID NO:70)	Gi 34819 NT 1- 30
8605	5' CCGGAATTCATAACAAGCTAATTTAAAAAACTTTTTGAA 3' (SEQ ID NO:71)	Gi 34819 NT 450 - 500
8559	5' CCCAAGCTTGACACAGACGGGAGGTACGGTATGGACGTCT 3' (SEQ ID NO:72)	Gi 33100 NT 460-490
8562	5' CCGGAATTCAAAAAATAAACTTGATTTATGAT GGTCAA 3' (SEQ ID NO:73)	Gi 33100 NT 705-734
8603	5' CCGGAATTCGCGGGTGACCTGCTTCCTGCCACCTGCTGT 3' (SEQ ID NO:74)	Gi 34819 NT 126-155
8692	5' CCGGAATTC AGTTAGTGCAGCCAAGCCCT 3' (SEQ ID NO:75)	Gi 33101 NT 301-320
8695	5' CCGGAATTCAAAAGGCAAGTGGACTTCGGTGCTTACCTG 3' (SEQ ID NO:76)	Gi 188910 NT 945-974
8697	5' CCCAAGCTT CAGCTCAGCTCAGTTCAGTTCAGCCCT 3' (SEQ ID NO:77)	Gi 188910 NT 4-30
8508	5' CCCAAGCTTATGCGAGGGTCTGGACGGCTGAGGACCCCC 3' (SEQ ID NO:78)	Gi 188910 NT 370-400
8696	5' CCGGAATTCATGCGGCAAGGGTTGCGGACCGCTGGCTGG 3' (SEQ ID NO:79)	Gi 188910 NT 715-744
8694	5' CCGGAATTCGCCCAGCCCAGCCTAGCTCA 3' (SEQ ID NO:80)	Gi 33101 NT 770-790
8691	5' CCCAAGCTTTTATCAACTGCTAGTTTGTG 3' (SEQ ID NO:81)	Gi 34819 NT 361-381
9090	5' CCGGAATTCAGGGCTGAACTGAACTGAGCTGAGCTG 3' (SEQ ID NO:82)	Gi 188910 NT 1-30
9879	5' CGGCTGATATCTGGGAGCCTCTGTGGATTTTCCGA 3' (SEQ ID NO:83)	Gi 33100 NT 1-24
9805	5' AGCCGGATATCGCCCAGCCCAGCCTAGCTCA 3' (SEQ ID NO:84)	Gi 33101 NT 770-790

0002	5' GAAAGTTAAATGGGAGTGACCCAG 3' (SEQ ID NO:85)	GI 29502084 NT 962860 - 962884
0021	5' GAGTGACCATCGCACCCCTTGACAG 3' (SEQ ID NO:86)	Gi 29502084

*Identification of rearranged VDJ segment in RAMOS RA-1*

- 5 The sequenced VH allele for RAMOS RA-1 was identified as VH<sub>4-34</sub> (DP 63) using V-Base (<http://www.mrc-cpe.cam.ac.uk/vbase>). A schematic diagram of the gene targeting region is shown in Figure 3 and the genomic sequence is set out in SEQ ID NO:1. The immunoglobulin heavy chain promoter shown in Figure 3 corresponds to nucleotides 4852 - 5190 of SEQ ID NO:1. The leader sequence (including intron 1) shown in Figure 3 corresponds to nucleotides 5191 - 5329 of SEQ ID NO:1. The VDJ segment shown in Figure 3 corresponds to nucleotides 5330 - 5708 of SEQ ID NO:1.

15 The consensus nucleotide sequence differs from the published VH<sub>4-34</sub> sequence (V - Base) by six nucleotides only (C<sub>68</sub>->G, C<sub>72</sub>->T, C<sub>228</sub>->G, T<sub>232</sub>->C, C<sub>244</sub>->T, C<sub>248</sub>->A) of which four were coding changes (A<sub>23</sub>->G; N<sub>76</sub>->K, F<sub>78</sub>->L, T<sub>83</sub>->N). The sequence was further classified as VH<sub>4-34</sub> subgroup 2 (Journal of Molecular Biology, 1987, Vol 195, 761-768) using the Kabat database (Kabat et al., 1991, Sequences of proteins of immunological interest, vol 1, 5<sup>th</sup> edition). The C<sub>68</sub>-> G mutation in framework 1 and N<sub>76</sub>- > K mutation in framework 4 were unique to RAMOS RA-1 and occurred in otherwise conserved residues in the other four members of the group (Lee et al. 1987, Journal of Immunology, 142, 4054-4061, Sanz et al. 1988, Clinical Experimental Immunology 71, 508-516).

25 The nucleotide sequence generated from RAMOS DNA corresponding to the D allele differed significantly from the published alleles in V Base. Although no significant homology was identified, the closest related sequence similarity (and sequence length) was to D<sub>3-16</sub> (Corbett, S et al, Journal of Molecular Biology, 270, 587-597).

30 The sequenced JH allele for RAMOS RA-1 was identified as JH6b (Mattila et al. 1995) using V-Base. No nucleotide base changes were detected between the consensus sequence and the published JH<sub>6b</sub> sequence.

*Sequencing of the 3' region downstream of the rearranged VH allele*

The homologous sequence downstream of the site of integration chosen for the vector corresponds to a ~ 3kb region between the rearranged VJD genes through to the mu enhancer of the human immunoglobulin heavy chain locus of the RAMOS cell line (corresponding to sequence gi 29502084 nucleotides 960091 – 962947).

The region 3' downstream of the rearranged VDJ segment was sequenced using methods previously described. Primers were designed based on published sequences, human immunoglobulin heavy chain enhancer on chromosome 14 (gi 34819), human J6 to enhancer DNA of the immunoglobulin heavy-chain gene (gi 33100), human (AW-Ramos) translocated t(8;14) c-myc oncogene, exon 1 (gi 188910) and human mu switch DNA of the immunoglobulin heavy-chain gene locus (gi 33101)(Table 5).

Sequences were analysed using BLAST program (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>) and assembled in Clone Manager Suite 7 (Scientific and Educational Software). The assembled sequence is set out as nucleotides 5709 to 8634 of SEQ ID NO:1. This sequence shares 98 % similarity with the published sequence gi 29502084 nucleotides 960091-962947.

*Cloning of the 3kb fragment downstream of the rearranged VH allele from genomic DNA*

The 3 kb fragment was amplified from genomic DNA extracted from RAMOS RA-1 cells using Platinum Pfx DNA polymerase (Invitrogen, CA, USA). The PCR reaction was the same as previously described except the forward primer 9779 (5' CCGCTCGAGTGGGAGCCTCTGTGGATTTTCCGA 3') (SEQ ID NO:111) and reverse primer 9801 (5' TGACCGGACGTCGCCAGCCCAGCCTAGCTCA 3') (SEQ ID NO:112) were used and the cycling conditions were as follows ; one cycle of 94°C for 15 seconds, fifteen cycles of 94°C for 10 seconds, 68°C for 2 minutes, 15 cycles of 94°C for 10 seconds, 68°C for 2 minutes with and an extra 15 seconds added each cycle, and one cycle of 72°C for 7 minutes. A second PCR reaction was performed using 0.5 / 20.0 µl of the first PCR reaction as a template to gain sufficient DNA for detection using ethidium bromide.

The 3 kb fragment was cloned into pPCRScrip using the PCR-Script Amp cloning kit (Stratagene, Texas, USA) at the Srf I site. The resulting construct was referred to as 3kbPCRScrip 10-1-3 (5822 bp). This construct was sequenced using primers in Table 5.

5

The size and GC rich stretches present in the 5 kb and 3 kb homology regions gave rise to structural regions which caused difficulties in cloning and thereafter problems with stability of the cloned regions.

## 10 Example 2: Design and construction of a vector for integration into the rearranged VH allele, VH4-34 in RAMOS RA-1

### *1) Construction of Vector For Integration*

15 A 3 kb fragment, containing sequence homologous to the region downstream of the rearranged allele VH<sub>4-34</sub> was amplified from the construct 3kbPCRScrip 10-1-3 with the forward primer 9879 (5' CGGCTGATATCTGGGAGCCTCTGTGGATTTTCCGA 3') (SEQ ID NO:83) and the reverse primer 9805 (5' AGCCGGATATCGCCCAGCCCAGCCTAGCTCA 3') (SEQ ID NO:84) using  
20 Platinum Pfx DNA polymerase (Invitrogen, CA. USA). PCR products were purified using QIAquick PCR Purification Kit (Qiagen<sup>TM</sup>) according to manufacturer's instructions then digested with EcoRV and ethanol precipitated. Digested fragments were ligated into construct 5kbPCRScrip 15a-7 containing the 5 kb sequence upstream of the rearranged VH allele. The DNA was transformed into Escherichia coli and grown  
25 at 37 °C overnight.

Bacterial colonies were screened by Southern blotting and probed using <sup>32</sup>P labeled oligonucleotides 8604 (5' CCCAAGCTTCGGCCCCGATGCGGGACTGCGTTTTGACCA 3') (SEQ ID NO:70)  
30 to detect the 3 kb fragment and a pool of labeled oligonucleotides 8687 (5' CCCAAGCTTCATGTTCCACGCATTACGTC 3') (SEQ ID NO:54), 8440 (5' CCGGAATTCAATTTGAGATTGTGTGTGAGATCTCAGGAG 3') (SEQ ID NO:38) and 8472 (5' CCGGAATTCGTTGGGTTCCCAGTGTAGGTGATGATCCAT 3') (SEQ ID NO:44) to detect the 5 kb fragment. Colonies that were positive for both  
35 fragments were subcultured and DNA was extracted using QIAprep Miniprep kit (Qiagen<sup>TM</sup>). Clones were analysed by diagnostic restriction enzymes and PCR for each



fragment and sequenced using an ABI 373 DNA sequencer with T7 and T3 primers. This new construct is referred to as 3kb15a-7- 4T is 10 826 bp long (Figure 4). The sequence of is shown in SEQ ID NO:87.

5     2) *Transfection of 3kb15a-7-4T into RAMOS and screening for integration*

Clone 3kb15a-7-4T (5 µg) is transfected into 3 x 10<sup>6</sup> RAMOS RA-1 cells using the following protocol ; cells are centrifuged to remove spent media then resuspended at 1 x 10<sup>6</sup> cells / ml in RPMI containing DEAE Dextran (25 µg / ml). Resuspended cells (1  
10 ml) are transferred into electroporation cuvettes (4 mm gap) and DNA is added. The cuvette containing the cell / DNA mixture is then incubated at 37°C for 10 minutes. Cells are then pulsed twice at 550 V and 25 µF capacitance, using a Gene Pulser (BioRad). Following a 10 minute incubation on ice, the cells are removed from the cuvette and transferred to T<sub>25</sub> flasks containing 9 ml of RPMI + 15 % FCS. Flasks are  
15 incubated at 37°C for at least 24 hours before the efficiency of transfection is assessed. Mock transfected cells are used as controls.

Three days after transfection, cells are stained for surface IgM and sorted by flow cytometry into an IgM positive population and IgM negative population. Prior to this  
20 experiment the background level of IgM negative cells is determined.

Genomic DNA is extracted from IgM negative cells using the Genoprep DNA isolation kit (Scientifx, Australia) according the manufacturers' instructions. The DNA is then digested using Xba I enzyme and run on a 0.6 % agarose gel. DNA is transferred onto  
25 nitrocellulose membrane using standard methods for Southern blotting and probed with <sup>32</sup>P labeled oligonucleotides 0041 (5' GACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGG 3') (SEQ ID NO:88) and 0042 (5' GACCTCCTGCACAAGAACGGTACCGGGCTAGAGCGGCCGCCA 3') (SEQ ID  
30 NO:89) that span the junction regions. A probe against the middle of the sequence that would be integrated, 9403 (5' CAGTGCTGCAATGATACCGCGAGAC 3') (SEQ ID NO:90) is also used.

The following patterns of radioactive probe binding to DNA extracted from cells  
35 transfected with i) vector only, no radioactive signals ii) the construct containing the 3kb15a-7 4T shows radioactive signals with all three radioactive probes as is expected

when the 5kb and 3kb recombination occurs with the chromosomal DNA integrating the middle sequence; the binding of the radioactive 9403 probe shows this iii) DNA from untransfected cells show no binding with the 9403 radioactive probe, iv) the lanes on the agarose gel with the plasmid 3kb15a-7-4T show radioactive signal with all probes. Obtaining this pattern i-iv) is indicative of 3kb15a-7-4T integration into the host cell genome.

Example 3 : Design and construction of a vector for integration into the rearranged VH allele, VH<sub>4-34</sub> in RAMOS RA-1 and mutation of the asFP499 gene

10

The components of the vector for integration are as follows:

i) Construct 5 kb in PCRScript 15a-7 is modified by removing the Nae I – Xho I fragment which effectively deletes an additional Kpn I site. This new construct is herein referred to as 5kbPCRScript minus Nae I – Xho I and is 7608 bp in length.

15

ii) The cloned gene for asFP499, which is a fluorescent protein isolated from the sea anemone *Anemonia sulcata*, was obtained from J. Wiedenmann (University of Ulm, Germany) in the plasmid pQE32 (Qiagen, CA. USA). Four sequential PCR reactions were performed to introduce restriction sites Kpn I and Not I at the 5' and 3' ends of the asFP499 gene respectively and two C-terminal flag tags with a Sal I site at the 3' end of the second flag tag. This final product (~770 bp) is subcloned into pPCRscript (Stratagene, Texas, USA) and herein is referred to as targetPCRScriptasFP499-1.

20

iii) The gene encoding thymidine kinase is amplified by PCR and restriction sites Hind III and Cla I are introduced at the 5' and 3' end of the gene respectively. This product (1260 bp) is subcloned into pPCRscript (Stratagene, Texas, USA) and herein is referred to as construct TKPCRscript -64.

25

iv) pMC1neo Poly A (3800 bp) was obtained from Stratagene (Texas, USA).

30

These components are assembled in the following order:

Constructs targetPCRScriptasFP499-1 and 5kbPCRScript minus Nae I – Xho I are digested with Kpn I and Sal I and run on 1.0 and 0.9 % agarose gels respectively. The desired products (~7608 bp and ~770 bp) are cut out and DNA extracted using

35

QIAquick gel extraction kit (Qiagen™). The asFP499 gene (~770 bp) is ligated into 5kbPCRScripT minus Nae I – Xho I (~7608 bp) to create 5kb-asFP499PCRScripT minus Nae I – XhoI (8289 bp).

5 Constructs TKPCRScripT -64 and 5kb-asFP499PCRScripT minus Nae I – XhoI are subsequently digested with Cla I and Sal I and run on agarose gels and DNA is extracted as described above. The asFP499-5kb fragment (~5770 bp) is ligated to the TKPCRScripT-64 backbone (~ 4737 bp) to generate TK-5kb-asFP499 PCRScripT (10464 bp).

10

The TK-5kb-asFP499 cassette (~7558 bp) is digested out of TK-5kb-asFP499 PCRScripT with Sal I and Hind III and ligated into pMC1neo Poly A, cleaved with Sal I and Hind III (3837 bp). This new construct is designated KW 1.

15 Finally, 3kbPCRScripT 10-1-3 and KW1 are digested with restriction enzymes Xho I and Aat II and gel purified as described above. The 3kb fragment is ligated into the KW1 backbone (~10868 bp) yielding the final integration vector designated KW2 (13722 bp) (Figure 5). The sequence of integration vector KW2 is set out in SEQ ID NO:91.

20

The asFP499 gene was deleted from vector KW2 to generate vector KW3 (Figure 6). The sequence of integration vector KW3 is set out in SEQ ID NO:110. It will be appreciated that any target nucleic acid molecule of interest can be inserted into this vector for use in the affinity maturation process of the present invention.

25

#### Example 4: Optimal culturing conditions for RAMOS RA-1 cells

30 Optimal growth conditions for RAMOS cells were determined by performing growth curve experiments using different supplements to the medium and different seeding concentrations. RAMOS cells were seeded at  $1 \times 10^4$ ,  $5 \times 10^4$ ,  $1 \times 10^5$ , and  $2 \times 10^5$  (cells / ml) and cultured as 25 ml cultures in either RPMI medium with 10 % heat inactivated FBS and 1mM sodium pyruvate or RPMI medium with 15 % heat inactivated FBS, 1 mM sodium pyruvate and 50 % conditioned medium. Every 24  
35 hours, 1 ml samples of cells were taken and the number of viable cells was determined using the Vi-Cell Viability Analyser (Beckman Coulter, CA, USA,). The results

showed that RAMOS cells had a lag phase of 48 hours regardless of the seeding concentrations. Cultures reach an exponential growth phase at a density of  $0.25 - 0.5 \times 10^6$  cells / ml RPMI medium with 10 % heat inactivated FBS and 1 mM sodium pyruvate whereas cells growing in RPMI medium with 15 % heat inactivated FBS, 1 mM sodium pyruvate and 50 % conditioned medium did not enter an exponential phase. The optimal seeding rate for RAMOS was  $1 \times 10^5$  cells / ml.

Mycoplasma infection can affect transfection rates, therefore we tested RAMOS RA-1 monthly. Cells were tested using 4', 6-diamidino-2-phenylindole (DAPI) staining in which all DNA is stained specifically with this very bright dye. If mycoplasma is present small bright specks of dye are seen in the cytoplasm. Cells were fixed onto glass slides and viewed under a 100 X objective with a UV filter system. Cells were negative for mycoplasma over a period of 12 months.

#### Example 5: RAMOS RA-1 cell division rate

Transfection rates in RAMOS are affected by the viability of cells in culture. To identify the optimal time during the growth cycle to transfect RAMOS cells, we first determined the cell division rate. RAMOS cells were stained with cell-permeant fluorescein-based dye carboxyfluorescein diacetate succinimidyl ester (CFSE) and analysed by flow cytometry based on established techniques in Current Protocols in Cytometry 9.11.2.

Briefly, RAMOS cells in exponential log phase growth were washed and re-suspended in phosphate buffered saline at  $5 \times 10^6$  cells / ml. A volume of 2  $\mu$ l of 5 mM CFSE was added per ml of cells. Cells were incubated for 10 minutes at 37°C after which 5 volumes of ice cold RPMI + 10 % FBS was added. Cells were then incubated on ice for a further 5 minutes and washed three times in culture medium before transferring to flasks and culturing under normal conditions (see example 1). Initially cells were not analysed until 12 hours post staining to allow for the initial fluorescence decay. Thereafter cells were analysed by flow cytometry every 24 hours. The CFSE fluorescence was plotted against the number of cells analysed (Figure 7). Upon cell division, the dye is equally distributed between daughter cells, allowing the resolution of up to eight cycles of cell division by flow cytometry. In the case of using cultured cells that divide in concert, the resulting fluorescent distribution is halved per cell after

each division. Therefore, these results indicate that the cell population was dividing at least once every 24 hours.

Example 6: Optimisation of conditions for transfection of RAMOS RA-1 cells

5

*1) Construction of vector for expression of asFP499 in RAMOS RA-1*

Primers were designed to add a Xho I site to the 5' end of the asFP499 gene and two  
flag tags, two stop codons and a Xba I to the 3' end of the gene to allow cloning into  
10 pME18s. The primers used are 8934 (5'  
CCGCTCGAGATGTATCCTTCCATCAAGGAAACC 3') (SEQ ID NO:92), 8935 (5'  
TCTAGATTATTATTTATCATCATCATCTTTATAATCTTTATCATCATCATCTT  
TATAATCAGCGGCCGC 3' (SEQ ID NO:93); 8936 (5'  
CTAGTCTAGATTATTATTTATCATCATCATC 3') (SEQ ID NO:94), and 8398 (5'  
15 GTTATGTCCTAATTTCTGAAGGCACTTGGGAGTA 3') (SEQ ID NO:95). The  
cloned sequence was confirmed by DNA sequence analysis. The resulting construct,  
referred to as pME18sasFP499 (3693 bp) (Figure 8) (SEQ ID NO:8) was used for  
subsequent transfection of RAMOS cells.

20 *ii) Transfection of pME18sasFP499 into RAMOS RA-1 cells*

A number of different transfection reagents or methods can be used to transfect  
mammalian cells including Calcium Phosphate coprecipitation, Lipofectamine  
(Invitrogen), FuGENE6 (Roche, Germany), SuperFect (Qiagen, CA, USA), Effectene  
25 (Qiagen, CA, USA), GenePORTER (Gene Therapy Systems, CA, USA) and  
Metafectene (Biontex, Germany). Electroporation is another method commonly used to  
transfect mammalian cells. Electroporation can be carried out using different  
electroporators such as the Gene Pulser (BioRad, CA, USA) or the Electro Square  
Porator ECM 830 (BTX, Fisher Biotech, Australia) and various voltage and  
30 capacitance settings can also be used. Since RAMOS RA-1 cells are of B-lymphoid  
origin and it is known that lymphoid cells can be difficult to transfect, we optimised  
transfection of this cell line.

The efficiency of transfection of RAMOS RA-1 cells was monitored by flow  
35 cytometric analysis using an EPICS Elite (Beckman Coulter, CA, USA). Samples of  
transfected cells were stained with 1 µg / mL Propidium Iodode (PI). The live cell

population (based on forward and side scatter characteristics and PI staining) was gated and the percentage of Fluorescent Protein (FP) positive cells was assessed. FP expression was also assessed by fluorescence microscopy using an Olympus IX70 microscope and Olympus U-RFL-T burner.

5

In order to optimise the transfection process, a number of different transfection parameters were tested including: set voltage, set capacitance, amount of DNA, concentration of DEAE Dextran and analysis time post transfection.

10 Figures 9 show the results of a representative set of optimisation experiments. The combination of 550 V and 25  $\mu$ Fd resulted in the highest transfection efficiency. No significant effect on the transfection efficiency was observed using 1  $\mu$ g or 30  $\mu$ g of pME18sEGFP. The optimum time to analyse FP expression was between 24 and 36 hours post electroporation. DEAE Dextran varying from 25  $\mu$ g / ml to 1 mg / ml was  
15 tested and concentrations between 25  $\mu$ g / ml and 100  $\mu$ g / ml were optimal for transfection but concentrations above 500  $\mu$ g / ml were found to be toxic to the cells (data not shown).

20 Using the optimum conditions, transfection rates of between 0.5 % and 3.5 % were achieved with the average being approximately 1.0 %.

Figure 10 shows the comparison of RAMOS RA-1 cells transfected with pME18sEGFP, pME18sasFP499 or mock transfected. The level of expression of asFP499 in RAMOS RA-1 was assessed 24 hours post transfection and was found to be  
25 approximately 10 fold lower than that of EGFP.

#### Example 7: Quantitation of the number of IgM molecules on the surface of RAMOS RA-1 cells

30

It is known in the art that the loss of surface IgM may be used to monitor integration into the rearranged VH allele.

35 We therefore determined the number of natural IgM molecules on the surface of RAMOS cells. RAMOS cells (50  $\mu$ l) and Quantum Simply Cellular™ beads (Bangs Laboratories, IN, USA) (50  $\mu$ l) were separately incubated on ice for 1 hour with a

5 saturating amount (2.5 µg) of a mouse anti-human IgM monoclonal antibody (Southern Biotech, AL, USA) conjugated to Alexa Fluor 488™ (Molecular Probes, OR, USA). Cells and beads were then analysed by flow cytometry using an EPICS Elite (Beckman-Coulter). Fluorescence intensity (at 488 nm) was plotted against the number  
10 of cells or beads analysed (Figures 11a and 11b). Five distinct populations of beads (labeled B, C, D, E, G) which have defined numbers of anti-mouse IgG binding sites (0, 2000, 20 000, 46 000 and 68 000) were observed (Figure 11a). Figure 11b (H) shows a normal distribution of fluorescence intensity, ranging from 2 to 100, as expected for a population of cells. The mean fluorescence for each of these populations  
15 was plotted against the corresponding number of predetermined binding sites to generate a linear line graph (Figure 11c) from which we extrapolated the average number of IgM molecules on RAMOS cells. The mean fluorescence intensity was calculated as 23.5 which corresponded to  $1.25 \times 10^6$  molecules for RAMOS RA-1.

15 Example 8 : Monitoring cell surface IgM loss on RAMOS RA-1

RAMOS RA-1 cell surface IgM decreases with time in culture. We therefore established the rate of natural loss of surface IgM in order to use this characteristic as a marker for integration. To evaluate the percentage of the population that were IgM  
20 positive, RAMOS RA-1 cells were stained for IgM and analysed by flow cytometry.

Briefly, cells were washed and resuspended at  $1 \times 10^6$  cells / 100 µl. A sheep polyclonal antibody against human IgM (µ chain specific) FITC conjugated (Chemicon, CA, USA) (10 µl) was added to cells which were incubated on ice for 1 hour. Cells were  
25 then washed with 2 ml of cold wash buffer (PBS + 2 % FBS, 0.01 % sodium azide) and resuspended in 500 µl this buffer with 5 µl propidium iodide (1mg / ml) (Sigma-Aldrich, Australia). Cells were analysed by flow cytometry using an EPICS Elite (Beckman Coulter, CA, USA). Cells were gated on the basis of forward and side scatter and negative propidium iodide. In passage one of RAMOS RA-1 98.39 % of  
30 the cell population was positive for surface IgM (Figure 12a) by passage 14, 26.55 % of the cell population was positive (Figure 12b). This loss is attributed to either a high mutation rate or a growth advantage of IgM negative mutants. Therefore, RAMOS RA-1 cells were presorted to remove IgM negative cells and the IgM<sup>+</sup> cells quantitated prior to transfection as the IgM loss is used as a measure of integration.

To further investigate IgM loss on RAMOS, we quantitated the number of cell surface IgM molecules using the methodology described above. We observed that the average number of cell surface IgM molecules significantly decreased over time. In passage 4, RAMOS RA 1 cells possessed an average  $9.0 \times 10^5$  molecules of IgM on their surface, however by passage 16 the number of IgM molecules had decreased to  $4.1 \times 10^5$  molecules (Figure 12). Together these data indicate that the decrease in cell surface IgM is dependent on passage number.

We have also observed and quantitated differences in the rate of IgM loss between RAMOS strains, 'RAMOS RA-1' supplied by American Tissue Culture Collection and 'RAMOS' supplied by European Collection of Cell Culture (ECAC) (Figure 13). Together these data suggest that although IgM loss is a marker for integration reported widely in the literature, we have opted to use an antibiotic marker, such as neomycin as a positive selection marker and thymidine kinase as a negative selection marker for integration in our system.

#### Example 9: Surface display of asFP499 using the anchor domain of CD26

For cell surface display of the asFP499 gene product on RAMOS cells we used the transmembrane domain of CD26 (Tanaka, T et al., 1992, J. Immunol. 149 (2), 481-486) as an anchor.

Transmembrane domain of CD26

Accession No: M74777 (gi 180082)

Peptide sequence: MKTPWKVLLGLLGAAALVTIITVPVLLNK (SEQ ID NO:96)

Nucleotide sequence: 10 -100

5'ATGAAGACACCGTGGAAGGTTCTCCTGGGACTGCTGGGGTGCTGCTGCGC  
TTGTCACCATCATCACCGTGCCCGTGTTCTGCTGAACAAA 3' (SEQ ID  
NO:97)

This anchor sequence was added to the 3' end of the asFP499 gene, to provide an N-terminal anchor on the protein (CD26asFP499). This was achieved by sequential overlap extension PCR using primers that partially overlapped the existing DNA end and also added new sequence. A representation of this sequential overlap PCR is shown in Figure 14. This method can be used to add sequence to either the 3' or 5' end of a sequence. Primers are listed in Table 6.



Table 6: Primers used for addition of CD26 anchor sequence to asFP499

Primer	Sequence
9712	5'CGTGCCCGTGGTTCTGCTGAACAAAATGTATCCTTCCATCAAGGAAACCA3' (SEQ ID NO:98)
9713	5' CCGTGCCCGTGGTTCTGCTGAACAAAGTGTATCCTTCCATCAAGGAAACCA3' (SEQ ID NO:99)
9726	5'TGCTGCTGCGCTTGTCACCATCATCACCGTGCCCGTGGTTCTGCTGAACAAA3' (SEQ ID NO:100)
9727	5'GGAAGGTTCTCCTGGGACTGCTGGGTGCTGCTGCGCTTGTCACCATCATCA3' (SEQ ID NO:101)
9728	5'CCGGAATTCATGAAGACACCGTGGAAGGTTCTCCTGGGACTGCTGGG3' (SEQ ID NO:102)
9963	5'GACTAGTTTATTATTATCATCATCATCTTTATAATCTTTATCATCATC3' (SEQ ID NO:103)

- 5 The final PCR product was cloned into pME18s as described previously except that the restriction sites *SpeI* and *EcoRI* were used. The cloned sequence was confirmed by DNA sequence analysis. The resulting vector was designated pME18sCD26asFP and is shown in Figure 15. The sequence of pME18sCD26asFP is set out in SEQ ID NO:104.

#### 10 Example 10: Transfection and analysis of CD26asFP499

To demonstrate cell surface display of CD26asFP499, the plasmid pME18sasFP499CD26 was transfected into RAMOS RA-1 and control HEK 293T cells.

- 15 RAMOS RA-1 cells were transfected as previously described (Example 6). Transfections in HEK 293T cells, which were 60 % – 90 % confluent, were carried out using FuGENE6 (Roche, Germany) according to the manufacturer's instructions. 5 µg of DNA was used in each transfection and pME18sasFP499 was used as a positive control.
- 20

Efficiency of transfection was assessed by flow cytometry as previously described. Figure 16 shows the flow cytometry data for transfection of RAMOS RA-1 cells with

pME18sCD26asFP499. It can be seen that the efficiency of transfection with this vector in this cell line is very low (0.01 % corresponds to 4 positive cells in this case). The data for transfection of HEK 293T cells is shown in Figure 17. In this cell line, the transfection efficiency of pME18sCD26asFP499 is equal to that of pME18sasFP499 and the expression of both vectors is improved in this cell line. The mean fluorescence intensity of pME18sCD26asFP499 was lower than that of pME18sasFP499.

HEK 293 T cells transfected with pME18sCD26asFP499 were analysed by confocal microscopy using a Nikon C1 (Coherent Life Sciences, Australia). The majority of cells showed a diffuse fluorescence at their periphery indicating expression of asFP499CD26 at the cell membrane, whereas bright fluorescence was observed uniformly throughout control cells transfected with pME18sasFP499 (data not shown).

#### Example 11: Cloning of the coding region of AICDA (AID) gene into pME18s

Activation Induced Cytidine Deaminase (AID), the protein product of the differentiation specific AICDA gene has been shown to be a B-cell-specific factor required and essential for the processes of Class Switch Recombination (CSR) and Somatic Hypermutation (SHM) in B-cells. Its ectopic expression in a number of mammalian cell systems including non B-cell systems has shown the ability of AID protein to induce and/or enhance hypermutation (Martin *et. al* 2002, Okazaki *et. al* 2002, Yoshikawa *et. al* 2002).

#### *Extraction of total mRNA from RAMOS RA-1*

Cells were harvested and centrifuged at 1500 rpm and resuspended in PBS. Total cellular mRNA was extracted using a GenoPrep<sup>TM</sup> mRNA isolation kit (Scientifix, Australia) according to the manufacturer's instructions. Briefly, after removing the supernatant, 700 µl of Lysis and Binding solution was added to cells ( $1 \times 10^6$ ). This mixture was combined with 50 µl (250 µg) of GenoPrep<sup>TM</sup> mRNA magnetic beads and incubated at room temperature for 5 minutes. Beads were magnetically collected and washed with 500 µl of washing solution I. Beads were then washed twice with 500 µl of washing solution II. mRNA-bead complexes were resuspended in 20 µl sterile water and then incubated at 65 °C for 2 minutes. Beads were magnetically collected and the mRNA-containing supernatant was transferred to a new tube.

The human AICDA (AID) gene was amplified from RAMOS RA-1 total RNA using the Superscript One-step RT-PCR with platinum Taq kit (Invitrogen, CA. USA). The reaction included 1 X reaction mix, forward primer 9645 (5' ATG GACAGCCTCTTGATGAACCGGAGGA 3') (SEQ ID NO:105) 10pM, reverse  
5 primer 9646 (5' CAAAGTCCCAAAGTACGAAATGCGT 3') (SEQ ID NO:106) 10pM, template RNA (~150 ng), RT / Taq mix (1.0 µl) and sterile water in a final volume of 50 µl. Cycling conditions were as follows; one cycle of 55°C for 30 minutes, 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for one minute, and one cycle of 72°C for 7 minutes.

10 The RT-PCR product (596 bp) was amplified by PCR using Platinum Pfx polymerase (Invitrogen CA., USA) as previously described. This product was then cloned into pPCRScript using the PCR-Script Amp cloning kit (Stratagene, Texas, USA) at the *Srf* I site. The coding region of AID was then subcloned into the pME18s using *Xho* I and  
15 *Xba* I restriction sites with primers 9792 (5' CCCTCGAGATGGACAGCCTCTTGATGAACCGGA 3') (SEQ ID NO:108) and 9793 (5' GCTCTAGACAAAGTCCCAAAGTACGAAATGCGT 3') (SEQ ID NO:109). The PCR reaction and cycling conditions were as described above. These constructs were verified by sequencing as previously described. The DNA sequence of  
20 the coding region of the AICDA gene is set out in SEQ ID NO:107.

#### Example 12: Affinity maturation of an antibody fragment

25 The gene targeting vector KW2 is modified such that following integration and target nucleic acid expression, a chimeric protein consisting of an antibody fragment fused to an anchor is produced. This is achieved by using standard molecular biology techniques to clone the CD26 anchor sequence into KW2 and then inserting the sequence for the antibody fragment downstream of the CD26 anchor sequence. The resulting vector is called KW3.

30 RAMOS RA-1 cells are transfected with the gene targeting vector KW3, using the optimised protocol previously described. The cells are allowed to recover for 48 to 72 hours before G418 at 5 mg / ml and gancyclovir or FIAU are added to the media for 7 to 9 days. This results in the selection of stable transfectants and also allows time for  
35 mutation and surface display to occur. The live cell population, which consists of the stable transfectants, is sorted using flow cytometry and allowed to react with the

fluorescently labelled binding partner of the displayed antibody fragment. The cells with the highest fluorescence intensity (ie highest binding) are then single cell sorted into 96 well U bottomed plates containing 100  $\mu$ l of 50 % conditioned media and 50 % fresh growth media. Single cell sorting is achieved using the Autoclone function of the EPICS Elite (Beckman Coulter, CA, USA).

It is also possible to select the cells with the highest affinity gene products on the cell surface, by capturing cells with immobilised binding partner, and selecting for the highest affinity gene product by competitive elution.

If necessary, the cells can be cycled through the *in vivo* strategy (Figure 2). The single cells can be expanded to between  $1 \times 10^5$  and  $1 \times 10^6$  to allow further mutation to occur and then reacted with the labelled binding partner and single cell sorted again. This cycle of single cell sorting/expansion and mutation/re-selection can be carried out until cells displaying the molecule with the desired characteristic, in this case increased binding affinity, have been isolated. The DNA from these cells can be extracted and the mutated gene can be amplified by PCR. Alternatively, the RNA can be extracted and the gene amplified by RT-PCR. The amplified gene can then be inserted into an appropriate expression vector for high-level production of the affinity matured antibody fragment.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:

1. A method for producing and selecting a gene product with desired characteristics, the method comprising
  - (i) introducing into a hypermutating cell a target nucleic acid molecule encoding a gene product such that the target nucleic acid molecule is integrated into an immunoglobulin locus of the genome of the hypermutating cell;
  - (ii) culturing the hypermutating cell such that the target nucleic acid molecule undergoes hypermutation during DNA and/or RNA synthesis, giving rise to a population of cells expressing mutant gene products; and
  - (iii) selecting a mutant gene product with desired characteristics.
2. A method as claimed in claim 1 wherein the immunoglobulin locus contains a rearranged V gene.
3. A method as claimed in claim 1 wherein the immunoglobulin locus contains a rearranged VH gene.
4. A method as claimed in claim 1 wherein the immunoglobulin locus contains the rearranged VH<sub>4-34</sub> allele.
5. A method as claimed in any one of claims 1 to 4 wherein following integration of the target nucleic acid molecule into the immunoglobulin locus, the target nucleic acid molecule is operatively linked to a promoter.
6. A method as claimed in claim 5 wherein the promoter is an immunoglobulin heavy or light chain promoter.
7. A method as claimed in claim 5 or claim 6 wherein the promoter is endogenous to the hypermutating cell.
8. A method as claimed in claim 5 or claim 6 wherein the promoter is exogenous to the hypermutating cell.

9. A method as claimed in any one of claims 5 to 8 wherein following integration the initiation codon of the target nucleic acid molecule is located within 2 kb of the 3' end of the promoter.
- 5 10. A method as claimed in any one of claims 5 to 8 wherein following integration the initiation codon of the target nucleic acid molecule is located within 500 bp of the 3' end of the promoter.
- 10 11. A method as claimed in any one of claims 5 to 8 wherein following integration the target nucleic acid molecule is located downstream of the promoter and upstream of an intronic enhancer with or without matrix attachment regions and/or 3' enhancer.
12. A method as claimed in any one of claims 1 to 9 wherein the target nucleic acid molecule is introduced into the cell by way of an integration vector comprising a sequence homologous to a region of at least 500 bp upstream of a rearranged V allele and a sequence homologous to a region of at least 500 bp downstream of a rearranged V gene.
- 15 13. A method as claimed in any one of claims 1 to 12 wherein steps (ii) and (iii) are repeated.
- 20 14. A method as claimed in any one of claims 1 to 13 wherein the method comprises a further step to increase the rate of mutation of the target nucleic acid molecule.
- 25 15. A method as claimed in claim 14 wherein the further step is to increase the levels of expression of activation-induced cytidine deaminase (AID) within the hypermutating cell.
- 30 16. A method as claimed in any one of claims 1 to 13 wherein the mutant gene product is selected by way of an assay performed within the hypermutating cell.
17. A method as claimed in claim 16 wherein the assay performed within the hypermutating cell is a protein-fragment complementation assay (PCA).

18. A method as claimed in any one of claims 1 to 17 wherein the target nucleic acid molecule is linked to a sequence encoding an anchor molecule such that following expression, the mutant gene product is displayed on the surface of the hypermutating cell.

5

19. A method as claimed in claim 18 wherein the mutant gene product is selected by detecting binding of a binding partner to the mutant gene product.

20. A method as claimed in claim 19 wherein the hypermutating cells are labelled with a detectable marker such as a fluorescent dye and the binding partner is immobilized.

10

21. A method as claimed in claim 19 wherein the binding partner is labelled with a fluorescent tag.

15

22. A method as claimed in claim 18 wherein hypermutating cell(s) displaying the mutant gene product bound to the labelled binding partner are sorted using a flow cytometric technique.

23. A method as claimed in any one of claims 19 to 22 wherein the binding partner is selected from the group consisting of an antibody, receptor, transcription factor hormone, enzyme, cell surface molecule, DNA or RNA molecule.

20

24. A method as claimed in any one of claims 1 to 23 which further comprises the step of recovering the target nucleic acid molecule encoding the selected mutant gene product.

25

25. A method as claimed in claim 24 wherein the recovery involves amplification of the polynucleotide by PCR or RT-PCR.

30

26. A method as claimed in any one of claims 1 to 25 wherein the hypermutating cell is a mammalian, yeast, insect or bacterial cell.

27. A method as claimed in claim 26 wherein the hypermutating cell is a mammalian cell.

35

28. A method as claimed in claim 27 wherein the mammalian hypermutating cell is selected from the group consisting of RAMOS, BL2, BL41, BL70 and Nalm.

29. A gene product produced by a method as claimed in any one of claims 1 to 28.

5

30. A vector for targeted integration into an immunoglobulin locus of a hypermutating cell, the vector comprising a sequence homologous to a region upstream of a rearranged V gene of the hypermutating cell, a sequence homologous to a region downstream of a rearranged V gene of the hypermutating cell and a site for integration of a target nucleic acid molecule.

10

31. A vector as claimed in claim 30 wherein the region upstream of the rearranged VH gene of the hypermutating cell is a region within nucleotides 1 to 5190 of SEQ ID NO:1

15

32. A vector as claimed in claim 31 wherein the region is at least 500 bp within nucleotides 1 to 5190 of SEQ ID NO:1.

33. A vector as claimed in claim 31 wherein the region upstream of the rearranged VH gene of the hypermutating cell comprises nucleotides 191 to 5190 of SEQ ID NO:1.

20

34. A vector as claimed in any one of claims 30 to 33 wherein the region downstream of the rearranged VH gene of the hypermutating cell is a region within nucleotides 5709 to 8699 of SEQ ID NO:1.

25

35. A vector as claimed in any one of claims 30 to 34 wherein the downstream region is at least 500 bp within nucleotides 5709 to 8699 of SEQ ID NO:1.

36. A vector as claimed in any one of claims 30 to 35 wherein the vector further comprises a selectable marker.

30

37. A vector for targeted integration comprising a sequence as set out in nucleotides 1 to 12990 of SEQ ID NO:110.



38. A vector as claimed in any one of claims 30 to 37 wherein the vector further comprises a sequence encoding a signal and/or anchor molecule suitable for display of the gene product encoded by the target nucleic acid molecule.

# Alignment of human immunoglobulin heavy chain promoters for VH4 alleles

L24134.SEQ	1	-TAAGTGAATCCCTGGTGTGCTGAACCTCAAGTGAATGTTACATTA----	ACCTGTGTNCCAATCTGTTTCTACCTGGGAAAAGAGGANCAGACATA	
L24135.SEQ	1	-TAAGTGAATCCCTGGTGTGCTGAACCTCAAGTGAATGTTACATTA----	ACCTGTGTGCAATCTGTTTCTACCTGGGAAAAGAGGAGCCAGACATA	
L24136.SEQ	1	ATTCCAAATC-----TGCTTTGATCCATGATCACATTTGCTCCAGACAGCTCCCTCAGCACATTTCC-TACCTGGAAAGAAAGAGACTCTGGGTTTG		
L24137.SEQ	1	ATTCCAAATC-----TGCTCCCTGATCCAGATCACACTGATCTCCAGACAGCAATCCCTACCTACCTTCCCTACCTGGAAGAGAGGACTATGGGCTTG		
L24138.SEQ	1	ATCCCAAATC-----TGTCNTTGTATCCAGGATCACACTCTCTCAGACAGCTCCCTTACGACACATCTCTTACCTGGGAAAGAGAGACTCTGGGCTTG		
CONSENSUS		AT CCAAATC TGCTCTGTATCCAGATCACACTTATCTC CAGAGCAGCT CTTACGACATTTCTTACCTGGAAGAGAGGACTCTGGGCTTG		
L24134.SEQ	97	GTGAGTTGAGGCCCCAGGAAGATACTGAATTCAGAGGGCAGCCAGCATCTCTTC-CCAGGGAGAGTCTAAAAGACTGGGGCTCCCTCATCCCT		
L24135.SEQ	97	GTGAGTTGAGGCCCCAGGAAGATACTGAATTCAGAGGGCAGCCAGCATCTCTTC-CCAGGGAGAGCTCTAAAAGACTGGGGCTCCCTCATCCCT		
L24136.SEQ	96	GTGAGGGAGNCCACAGGAAGAGAACTGATTTCTCAGAGGGCAGCCAGCATACACCTC-CCAGGGTGAAGCCCAAAGACTGGGGCTCCCTCATCCCT		
L24137.SEQ	97	GTAAGGGAGGCCACAGGAAGAGAACTGATTTCTCAGAGGGCAGCCAGCATCTCTTC-CCAGGGCAAGCCCAAAGACTGGGGCTCCCTCATCCCT		
L24138.SEQ	97	GAGAGGGAGCCCCAAGAGAACTGATTTCTCAAAGGGCAGCCAGCATCTCTTC-CCAGGGTGAAGCTCAAAGACTGGGGCTCTCTCATCCCT		
CONSENSUS		GTGAGGGAGGCCCCAGGAAGAACTGATTTCTCAGAGGGCAGCCAGCATCTCTTC CCAGGG GAGGCCAAAAGACTGGGGCTCCCTCATCCCT		
L24134.SEQ	196	TTTCACCTGTCCATACAGAGGCACCCACATGCAATC-TCACTTAGGCACCCACAGAAACCAACACACATTTCTTAAATTCAGGGTCTCTGCTCAC		
L24135.SEQ	197	TTTCACCTGTCCATACAGAGGCACCCACATGCAATC-TCACTTAGGCACCCCAAGGAAACCAATCACACATTTCTTAAATTCAGGGTCTCTGCTCAC		
L24136.SEQ	195	TTTTACCTATCCATACAAAGGCACCCACATGCAATCCTCCTTAGGCACCCACAGGAAATGATACACATTTCTTAAATTCAGGGTCCAGCTCAC		
L24137.SEQ	196	TTTCACCTGTCCATACAAAGTCAACCGCCACATGCAATCCTCCTTAGGCACCTACAGGAAACCCAGCACACATTTCTTAAATTTGGGATCCAGCTCAC		
L24138.SEQ	196	TTTCACCTGTCCGTACAAAGCACCCACATGCAATCCTCCTTAGGCACCCACAGGAAAGCCACACACATTTCTTAAATTCAGG-TCCAACTCAT		
CONSENSUS		TTTCACCT TCCATACAAAGGCACCCACATGCAATCCTCCTTAGGCACCCACAGGAAACCCACACATTTCTTAAATTCAGGGTCCAGCTCAC		
L24134.SEQ	295	ATGGGAAATACTTTCTGAGAGTCTCTGGACCTCTCTGTGCAAGAAC		
L24135.SEQ	296	ATGGGAAATACTTTCTGAGAGTCTCTGGACCTCTCTGTGCAAGAAC		
L24136.SEQ	295	ATGGGAAGTCTTTCTGAGAGTCTCTGGACCTCTCTGCAAGAAC		
L24137.SEQ	296	ATGGGAAATACTTTCTGAGACTCATGGGCTCTCTGCAAGAAC		
L24138.SEQ	295	AAGGGAATGCTTTCTGAGAGTCTCTGATCTCATGTGCAAGAA		
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FIGURE 1

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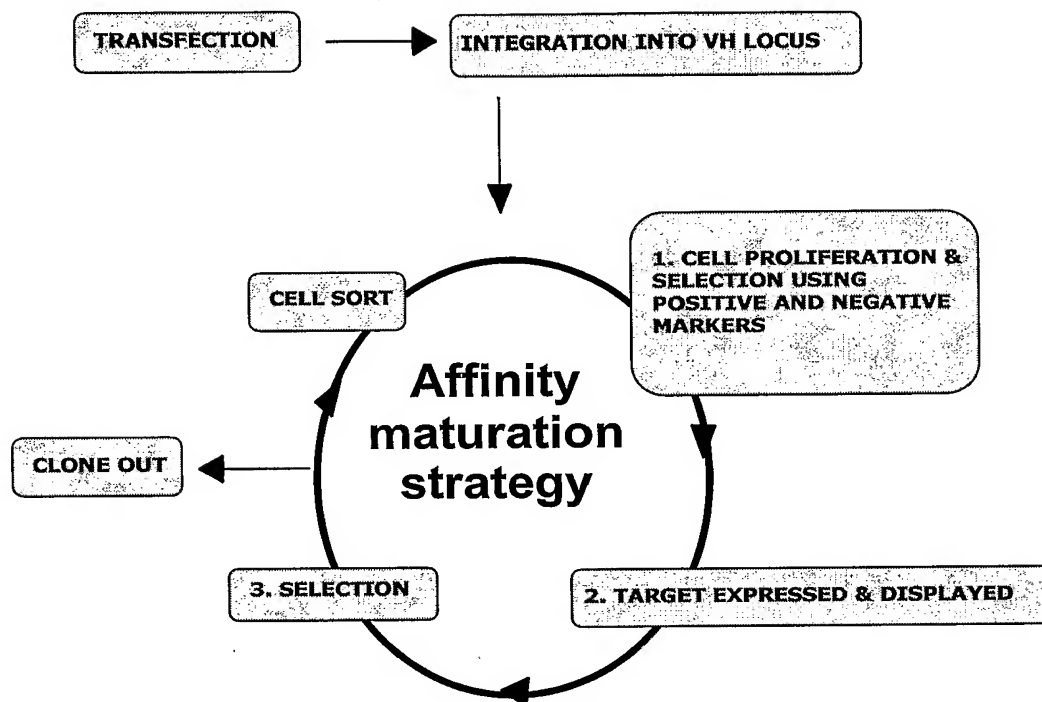
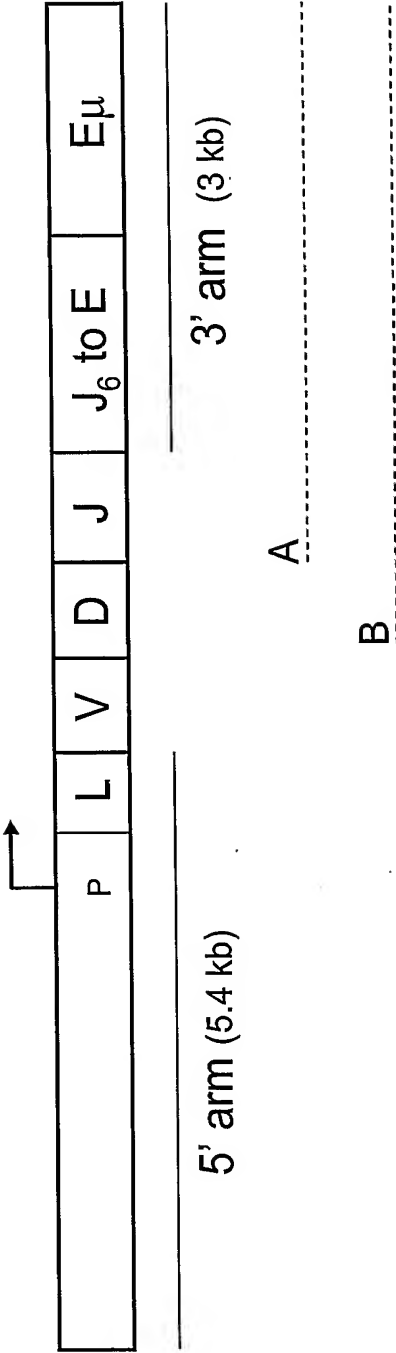


FIGURE 2



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FIGURE 3

P = VH4-34 promoter  
L = leader sequence  
VDJ = VH4-34 rearranged Variable, Diversity and Join gene regions  
J<sub>6</sub>-E = Ramos J6 to enhancer sequence  
E<sub>μ</sub> = Intronic enhancer region  
A = Potential 3' homology region  
B = Potential 3' homology region (preferred site for homologous recombination)

\* Not drawn to scale

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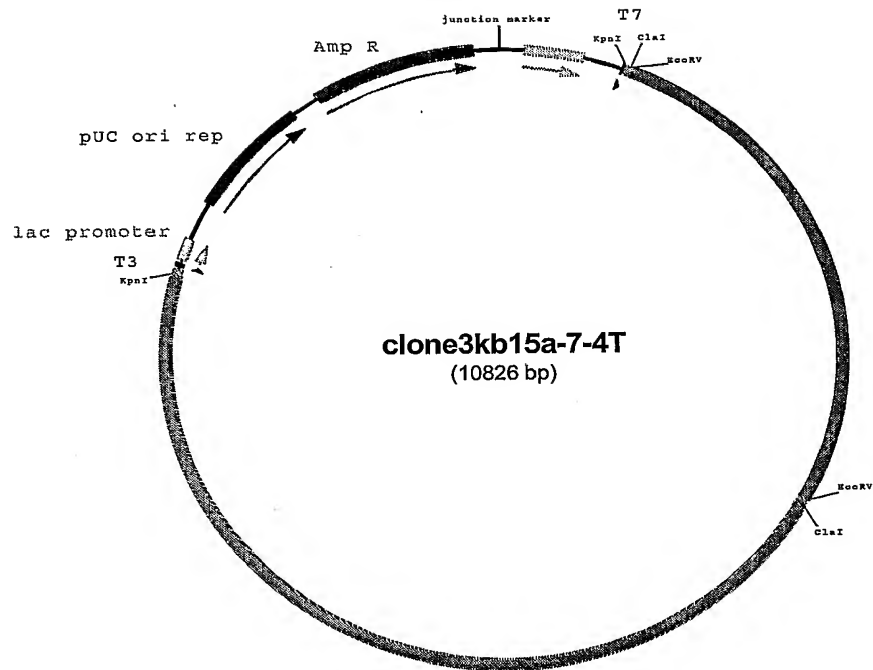


FIGURE 4

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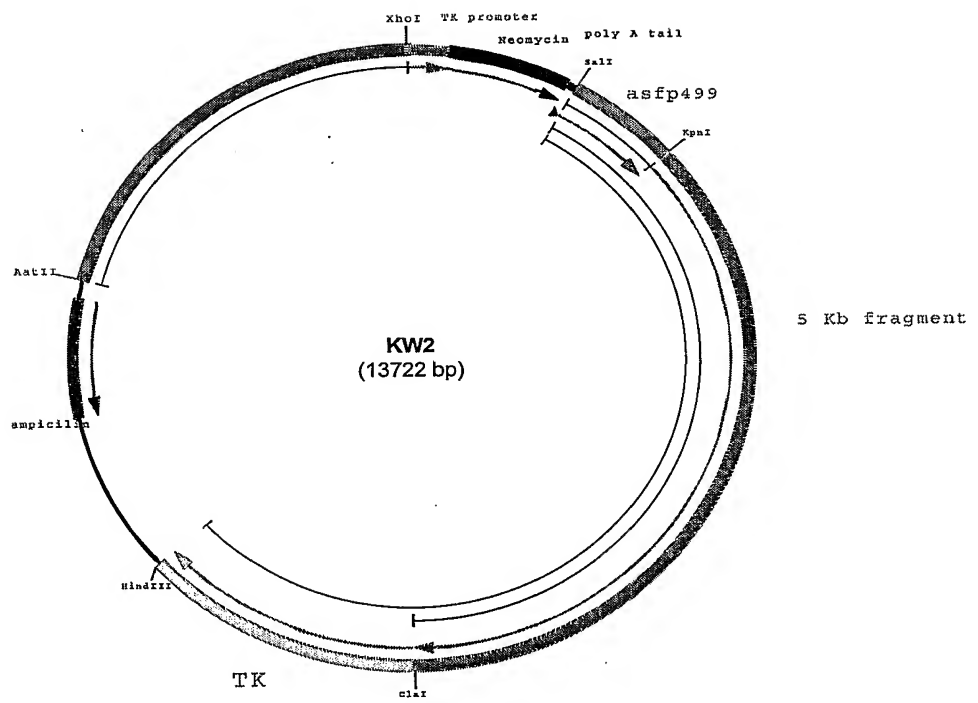


FIGURE 5

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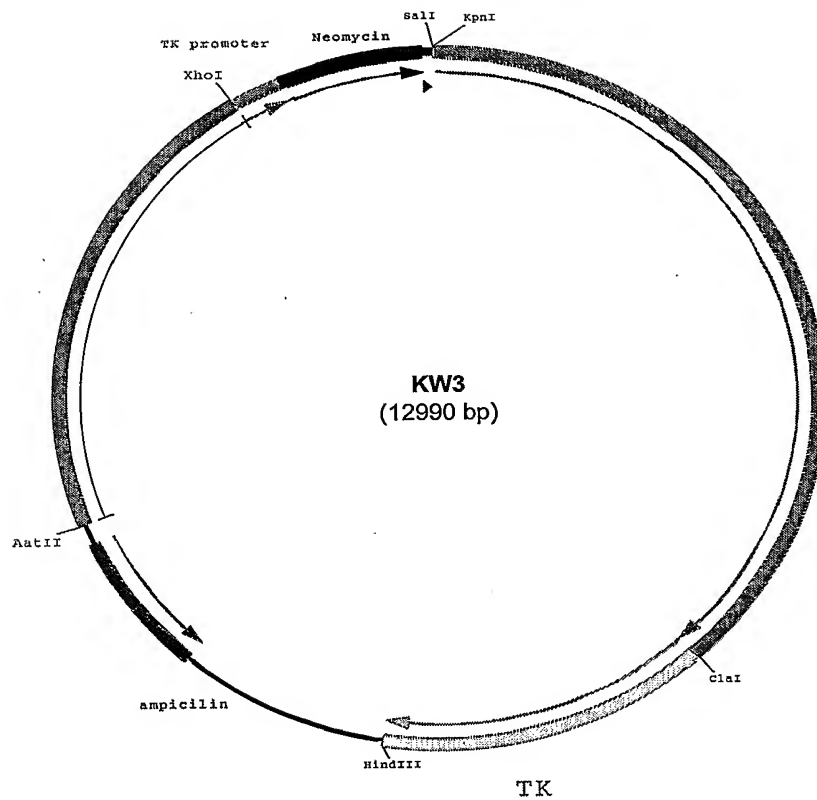
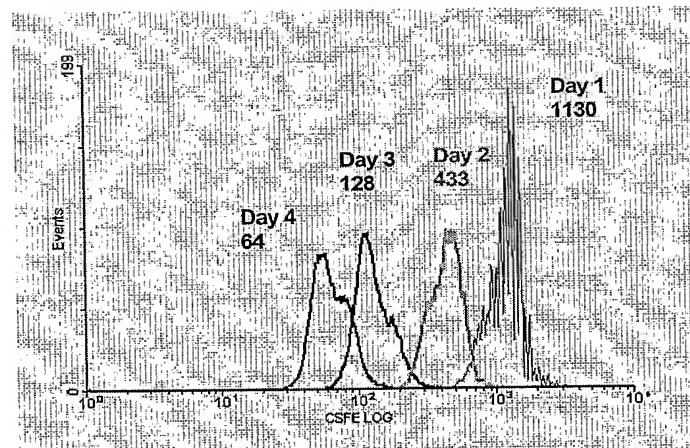


FIGURE 6

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Ramos cells stained with CFSE showing successive divisions on each day

FIGURE 7



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FIGURE 8

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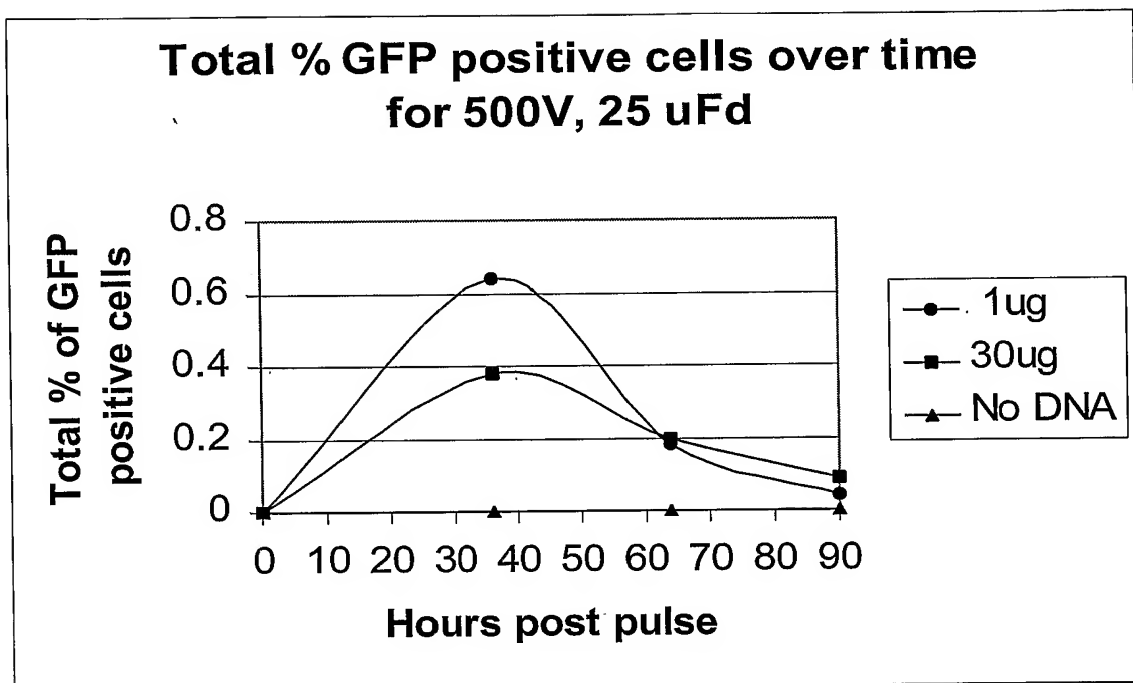
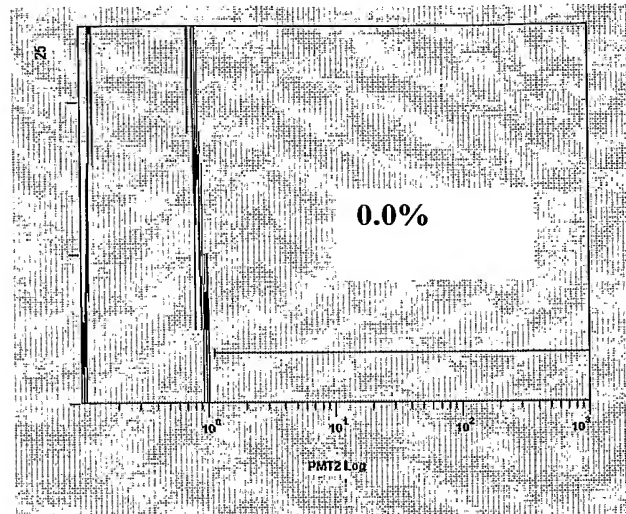
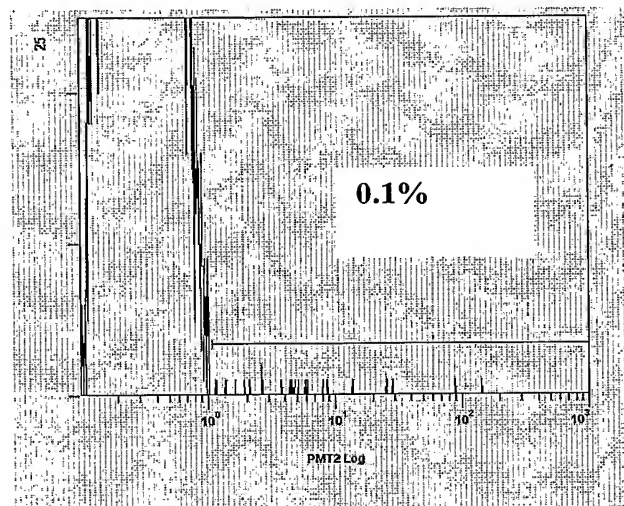


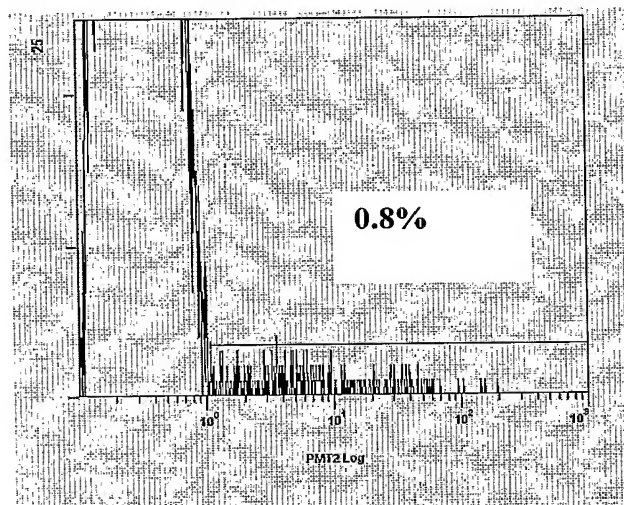
FIGURE 9

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**Mock transfected RAMOS (RA1)****pME18sasFP499 (5 $\mu$ g) transfected RAMOS (RA1)****FIGURE 10**

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**pME18seGFP (5 $\mu$ g) transfected RAMOS (RA1)**



**FIGURE 10 continued**

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Quantum Simply Cellular Beads stained with  
2.5 $\mu$ g mouse anti-human IgM-Alexa 488 per  
50 $\mu$ l beads

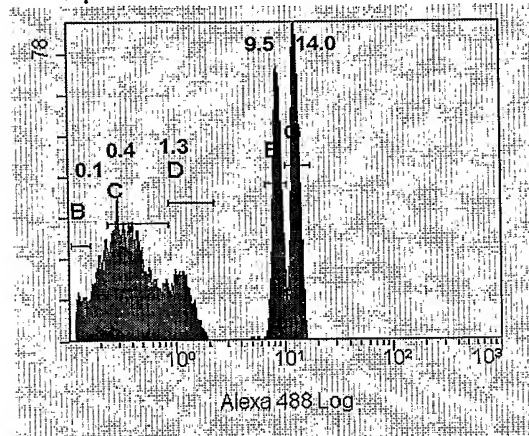


FIGURE 11A

Ramos cells stained with 2.5 $\mu$ g mouse  
anti-human IgM-Alexa 488 per 50 $\mu$ l cells

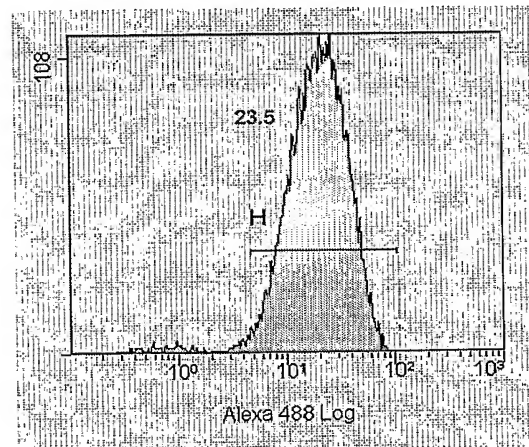


FIGURE 11B

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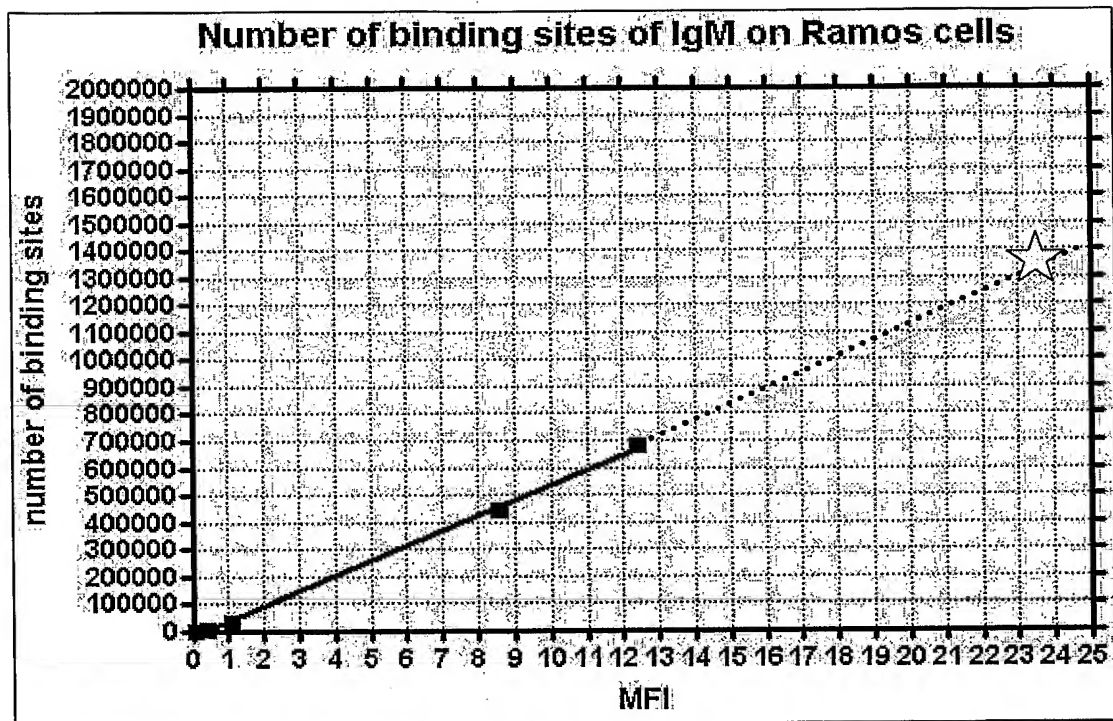


FIGURE 11C

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RAMOS RA1 p1

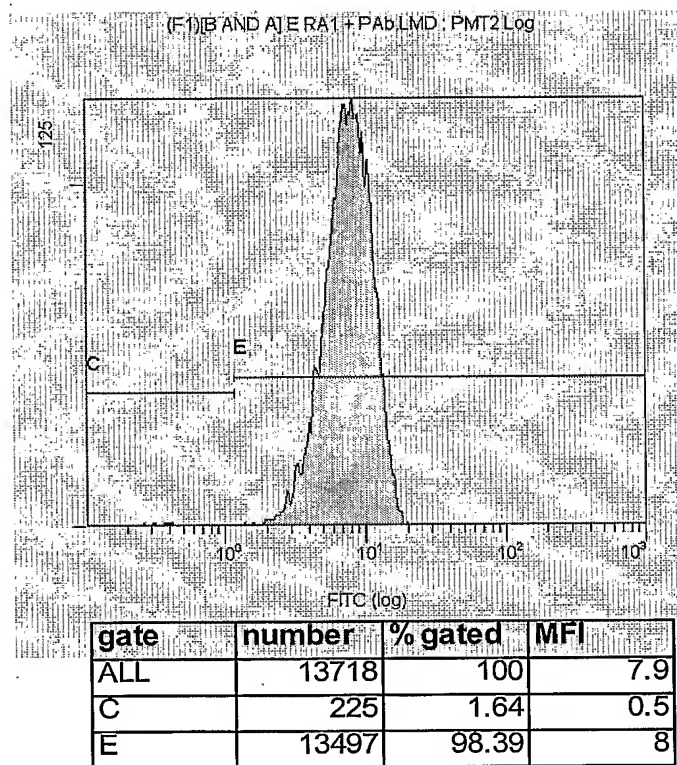


FIGURE 12A

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RAMOS RA1 p14

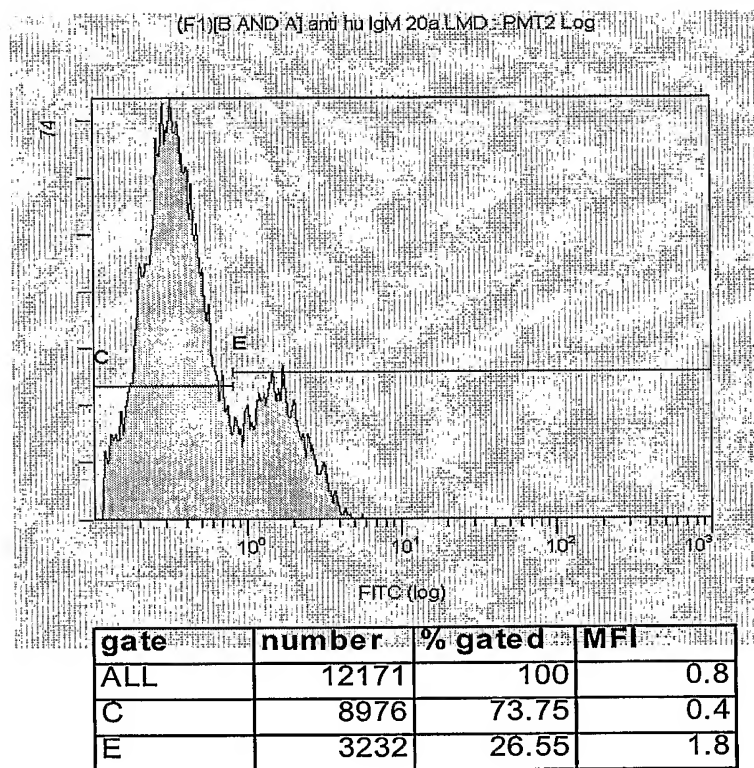


FIGURE 12B



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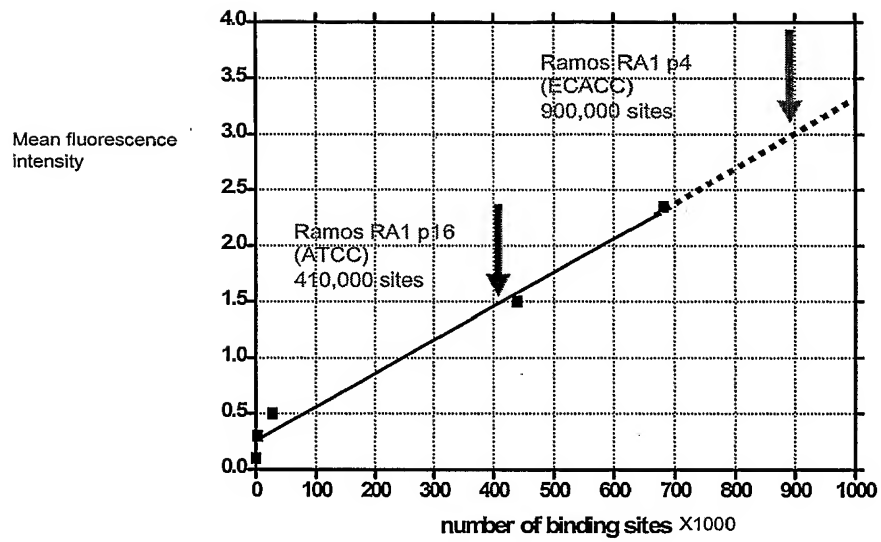


FIGURE 13

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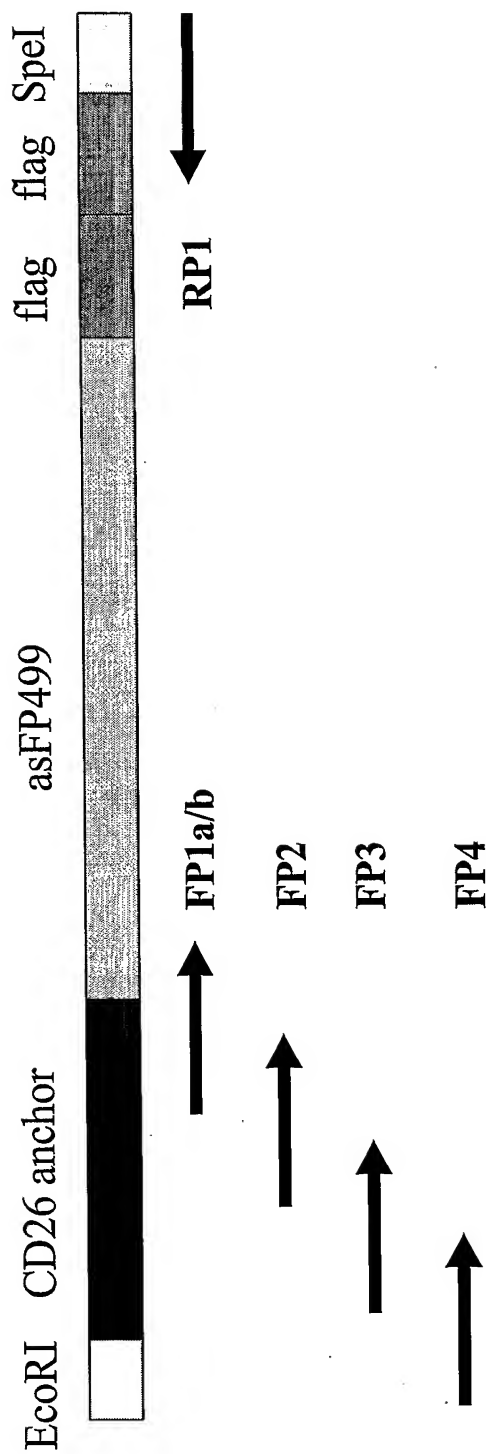


FIGURE 14

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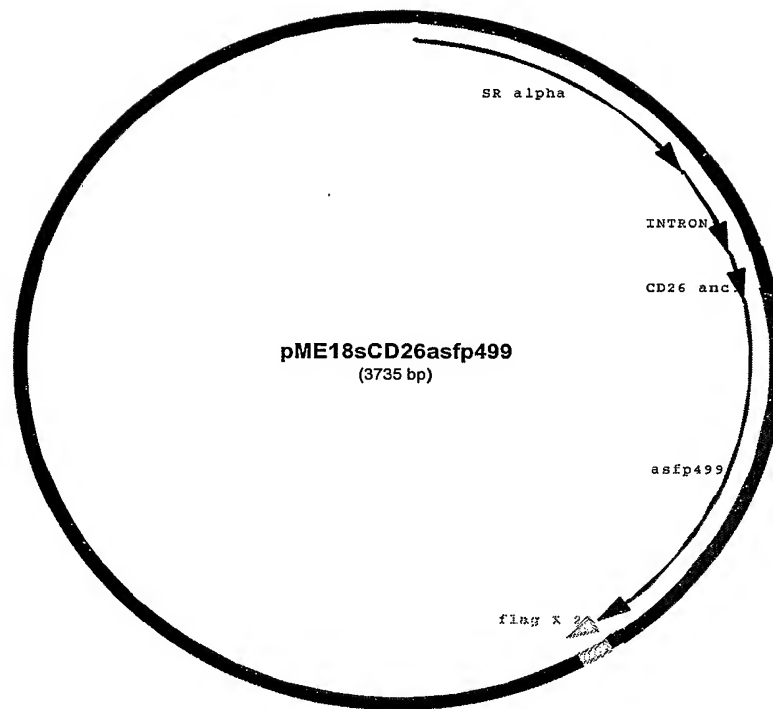
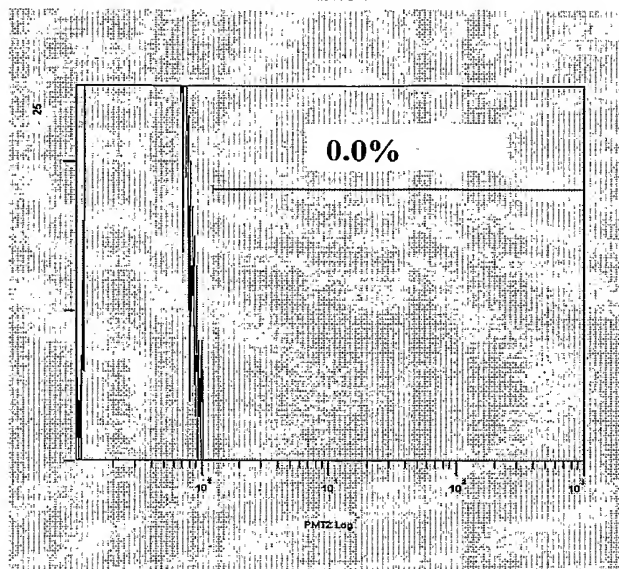
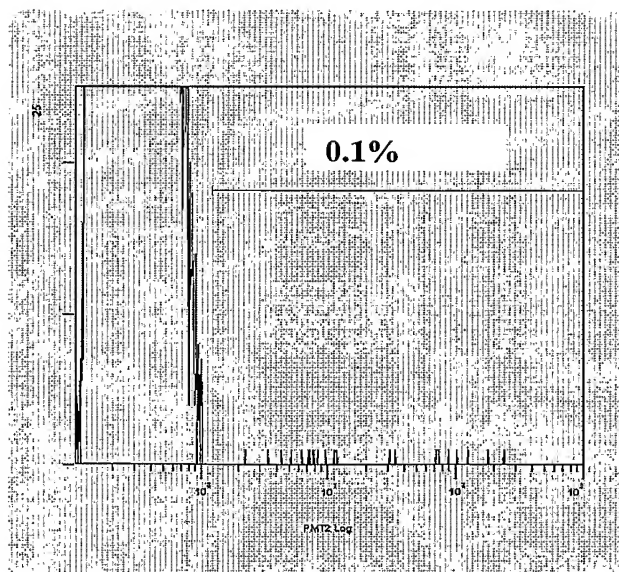
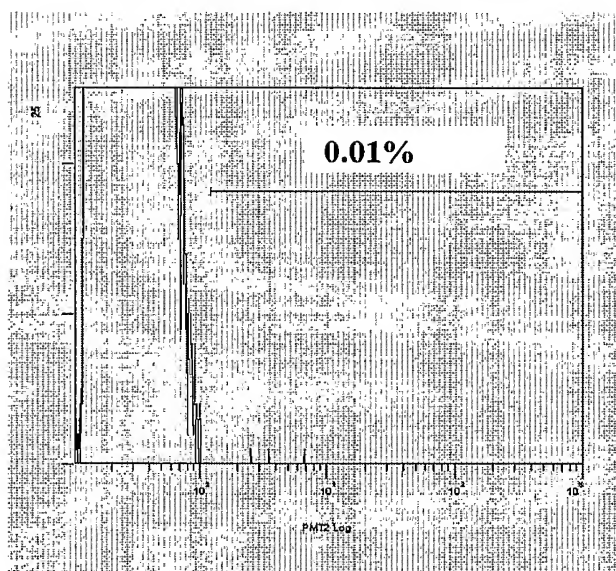


FIGURE 15

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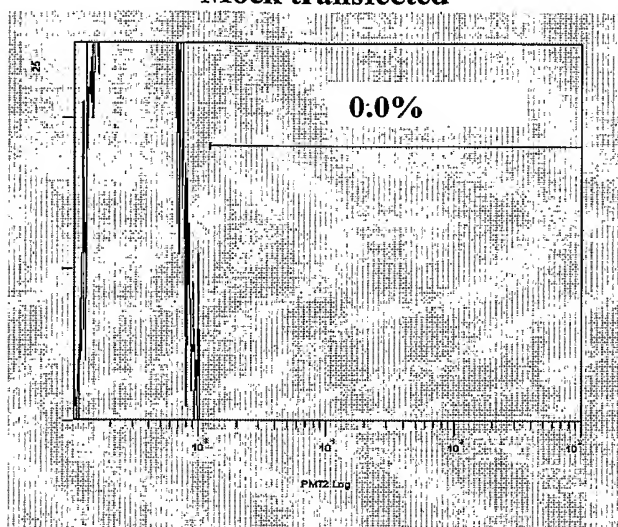
**Mock transfected****pME18sasFP499 (5μg)****FIGURE 16**

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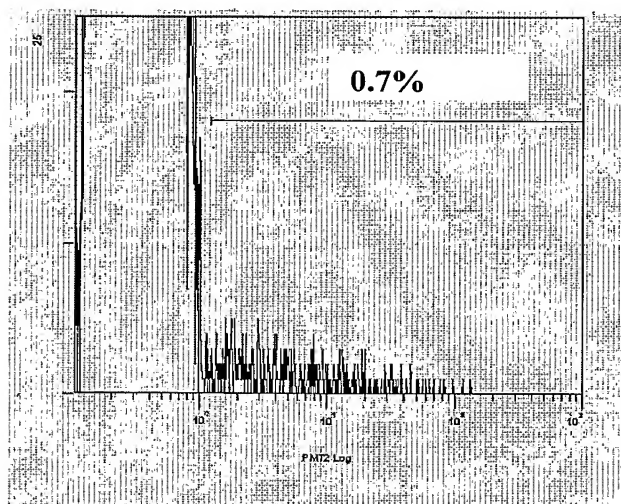
**pME18sCD26asFP499 (10 $\mu$ g)****FIGURE 16 continued**

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**Mock transfected**



**pME18sasFP499 (5μg)**



**FIGURE 17**

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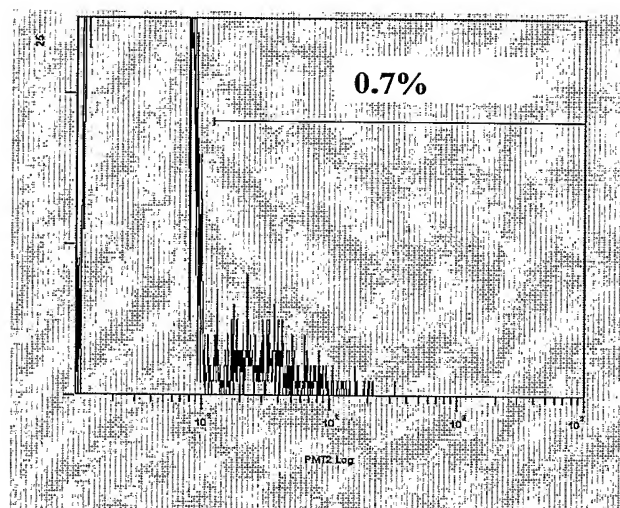
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FIGURE 17 continued

## SEQUENCE LISTING

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<151> 2002-12-18

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 aagaagtggg tttgaaacac tctgtccagc cccaccaaac cgaaagtcca ggctgagcaa 180  
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<213> Mus musculus

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<210> 18  
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acatgcaa at agggccctcc ctctactgat gaaaaccagc ccagccctga ccctgcagct 180  
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<210> 21  
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 ccctctgctg atgaaaacca gccagccct gaccctgcag ctctgggaga ggagccccag 180  
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 taaatagcat ccttcatgaa tatgcaaata actgaggtga atatagatat ctgtgtgccc 180  
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<210> 23  
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<400> 23

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Gly Leu Leu Thr  
 35

<210> 24  
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Cys Arg Thr Asn Tyr Gly Tyr Ser Ala Ala Pro Ser Leu His Leu Pro  
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<210> 27  
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 <212> PRT  
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<400> 27

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Gly Gly Ile Ser Leu Leu Val Gln Asn Thr Ser Trp Met Leu Leu Leu  
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 gccctggact tcatt 135

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<400> 31

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Gly Phe Gly Ala Val Ile Thr Val Val Val Ile Val Val Ile Ile Lys  
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Cys Phe Cys Lys His  
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31

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24

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24

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# INTERNATIONAL SEARCH REPORT

International application No.  
**PCT/AU2003/001697**

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>					
Int. Cl. <sup>7</sup> : C12N 15/10, 15/63					
According to International Patent Classification (IPC) or to both national classification and IPC					
<b>B. FIELDS SEARCHED</b>					
Minimum documentation searched (classification system followed by classification symbols) <b>SEE ELECTRONIC DATABASE BOX BELOW.</b>					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched <b>SEE ELECTRONIC DATABASE BOX BELOW</b>					
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Medline, CA, WPIDS: affinity maturation, somatic hypermutation, genetic vectors, ramos, bl2, bl41, bl70, nalm, vh, hypermutat?, cultur?, Ig, immunoglobulin, sequence ID 1.					
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>					
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.			
A	US-A-5885827 (Wabl, et al) 23 March 1999	30-37			
A	YELAMOS, J et al (1995) "Targeting of non-Ig sequences in place of the V segment by somatic hypermutation" <i>Nature</i> , 376:225-229.	1-29			
A	MARTIN, A. and SCHARFF, M. D. (2002) "Somatic hypermutation of the AID transgene in B and non-B cells" <i>Proceedings of the National Academy of Sciences USA</i> , 99(19):12304-12308.	1-29			
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <span style="margin-left: 100px;"><input type="checkbox"/> See patent family annex</span>					
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;"> <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 33%; vertical-align: top;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </td> <td style="width: 33%;"></td> </tr> </table>			<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>	
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>				
Date of the actual completion of the international search <b>3 February 2004</b>		Date of mailing of the international search report <b>- 9 FEB 2004</b>			
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer  <b>PHILIPPA WYRDEMAN</b> Telephone No : (02) 6283 2554			

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2003/001697

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	IRVING, R. A. et al (1996) "Affinity maturation of recombinant antibodies using <i>E. coli</i> mutator cells" <i>Immunotechnology</i> , 2:127-143.	1-29